EWE UNDERNUTRITION ALTERS COTYLEDON DEVELOPMENT AND FUNCTION

by

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DEDICATION

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ABSTRACT

Undernutrition is common for animals maintained on rangelands, particularly during periods of drought. Undernutrition during gestation can be detrimental to offspring development. The placenta is a known mediator for maternal-fetal nutrient exchange, and developmental impacts to the placenta during nutritional stress contribute to poor offspring outcomes. The objective of this study was to determine the impacts of maternal nutrient restriction on the development and function of placental cotyledons. Targhee ewes were allocated into two dietary treatment groups for the entirety of gestation. The control group (CON; n = 10) was fed to meet 100% of NRC requirements and the nutrient restricted group (NR; n = 11) was fed to meet 60% of NRC requirements, to mimic the nutritional stress experienced by range ewes during drought. At the time of lambing total placental weight, total cotyledon number, and lamb birth weight was recorded. Cotyledons (n = 3 / placenta) were removed from multiple locations, weighed, and processed for RNA-sequencing. Total placental weight, total cotyledon number, and lamb birth weight were not affected by maternal diet restriction ($P > 0.05$). Average cotyledon weight was greater in the CON group compared to the NR group ($P < 0.05$). Gene ontology (GO) and Kyoto encyclopedia of genes and genomes pathways (KEGG) analyses were performed to identify biological pathways involved in placental function and nutrient transfer. Cotyledons from NR singletons vs CON singletons expressed 470 differentially expressed genes (DEGs; $P < 0.05$; 187 up-regulated, 283 down-regulated). Gene ontology terms represented by DEGs include hormone activity ($P = 0.0003$) and antigen processing and presentation ($P = 0.0004$). KEGG pathways of interest include pyruvate metabolism, estrogen signaling, extracellular matrix receptor interaction, and antigen processing and presentation. There were also 426 DEGs ($P < 0.05$; 195 up-regulated, 231 down-regulated) in cotyledons of NR twins vs CON twins. Represented GO categories include heme binding ($P = 0.003$) and oxidoreductase activity ($P = 0.03$). These data show that prolonged nutrient restriction alters cotyledon development and gene expression indicating impacts to placental function. These changes in placental function likely mediate poor offspring developmental outcomes observed following gestational under nutrition.

CHAPTER ONE

INTRODUCTION

Reproduction is the first biological system that is sacrificed when adequate nutrition is lacking. Animal agriculture has a major role in Montana's economy, and $1.6 billion dollars was generated by animal production in Montana in 2021 (NASS, 2022). Montana ranks 8th in the United States for total sheep numbers (Association, 2023). The majority of these sheep in Montana are raised on native rangelands (NASS, 2022). Sheep provide multiple opportunities for producers to be profitable. Sheep can be grazed alongside cattle because they use forage sources that cattle cannot, such as noxious weeds and woody plants. Sheep are raised for meat, as well as fleece and wool products. Although sheep can use multiple forms of forage throughout the year the possibility of undernutrition is still cause for concern.

Montana poses unique challenges for livestock producers, such as drought and reduced growing season length. Drought is a common occurrence in Montana and across the country. During the summer in 2021, the U.S Drought Monitor classified over half the state of Montana as experiencing some level of drought. Furthermore, it classified the week of November 23rd, 2021, as being "the most intense period of drought", when 33.1% of Montana lands were experiencing exceptional drought conditions. Drought severely limits forage production and forage quality. Drought can be challenging for producers to not only find hay but also purchase and stockpile for the winter months. Another challenge is the growing season length. The growing season is measured from the last frost to the first frost of the year, which averages 135 days in Montana. The growing season normally begins around mid-May and continues until late

September/early October. Once the growing season is over, there is a risk that forage will not meet the nutritional requirements of gestating animals. The timing of breeding coincides with the end of the growing season in Montana; thus, ewes are typically bred later in the year to ensure lambing early in the spring.

Reproductive efficiency is a primary goal for livestock producers, and dam nutrition plays an important role in a successful breeding season. Maternal nutrient restriction is an issue that sheep producers frequently face. Without adequate nutrition during gestation there are consequences to fetal development and offspring health (Wu et al., 2004). These consequences lead to less productive lambs and can decrease their profitability. Profitability for sheep producers is influenced by, rate of gain, carcass, and fiber quality, developing replacement females, and ram fertility (Knott, R). These production traits can be impacted by insults that occur during fetal development. The formation of the placenta is a key factor in mitigating these insults. Insults applied during gestation include nutrient restriction, over nutrition, and the ingestion of toxins. The placenta is a highly specialized organ that allows for feto-maternal nutrient, gas, and waste exchange. All nutrients needed by the fetus and fetal waste products pass through this highly vascularized organ during pregnancy. The placenta acts as the mediator for nutrient partitioning. The placenta can overcome some levels of restriction through tissue and vasculature modifications; however, these adaptations are not enough to overcome prolonged and severe levels of restriction. The following sections discuss the development and function of the placenta and placental and fetal outcomes following maternal dietary stress.

CHAPTER TWO

LITERATURE REVIEW

Early embryo development

Ewes typically ovulate within 24-27 hours following the onset of estrus. During this time the oocyte is viable for roughly 25 hours post-ovulation (Kennedy, 2012). Once the oocyte is fertilized, and termed the zygote, it will experience the first cell division. Following the first cell division, symmetrical cell division continues until the embryo reaches the morula stage (Johnson, et. al., 2018). This early embryo is encased within the zona pellucida, the protective layer around the perimeter. The morula enters the uterus around day 3-4 post-fertilization, and the endometrium begins secreting growth factors to aid in embryonic growth and development. These growth factors in addition to glucose, vitamins, amino acids, nutrient transport proteins, and other compounds aid in the development of the offspring throughout pregnancy are termed "histotroph" (Bazer, et. al., 2011). At day 6 post-fertilization, the embryo, termed a blastocyst, begins to develop 2 distinct cell types: the inner cell mass (ICM) and the trophectoderm (Johnson, et. al., 2018). The ICM is comprised of cells that will later form the embryo proper and the trophectoderm is comprised of the precursor cells for the placenta. Trophoblast cells, which make up the trophectoderm, play a pivotal role in communication between the developing conceptus and the endometrium (Fritz et al., 2014; Godakumara et al., 2021). As the blastocyst continues to grow it will hatch from the zona pellucida. By day 11, the blastocyst (now referred to as a conceptus) is beginning to elongate because of rapid proliferation of the trophectoderm. This elongation process allows for increased surface area for placental attachment.

Maternal recognition of pregnancy (MRP) is the signal released by the conceptus once it enters the uterus (Bazer, 2013). This signals the uterus to continue to produce an environment conducive for growth and maintenance of pregnancy. Interferon-tau (INF-τ) is a hormone released by trophoblast cells and is the main messenger for the maintenance of pregnancy. INF-τ is first detectable in circulation around day 10 post-fertilization and its concentration peaks at the time of implantation, on day 16 (Raheem, 2017). Progesterone secretion by the corpus luteum (CL) is required for the maintenance of pregnancy until the placenta develops enough to take over hormone production. The MARP signal is involved in the maintenance of the CL. The pathway in which INF-τ maintains the CL has been a long-standing question of researchers. The release of oxytocin from the endometrium induces prostaglandin F2α (PGF2α) release, which is the hormone responsible for luteolysis. INF-τ blocks the receptors that oxytocin binds with on the endometrium (Spencer et al., 1995; Johnson et al., 2018). By preventing the binding of oxytocin to its receptors, PGF2α production is inhibited, which in turn prolongs the life of the CL (Spencer et al., 1995; Raheem, 2017; Johnson et al., 2018). Without the release of INF-τ, PGF2α is released into circulation and luteolysis occurs.

Trophoblast cells allow for the free-floating conceptus to adhere to the endometrium. This attachment occurs around day 16 post-fertilization and is when trophoblast binucleate cells (BNCs) begin to differentiate (Spencer, et. al., 2004). Total attachment of the conceptus occurs around day 22 of pregnancy. Following this, the placenta develops and takes over control of progesterone production and transfer of nutrients to the fetus (Spencer et al., 2004).

The Ruminant Placenta

The ruminant placenta is classified as syndesmochorial, meaning there are areas of the endometrium that erode away, allowing for more efficient transfer of nutrients (Johnson et al., 2018). Ruminant placentas form structures referred to as placentomes, which consist of the maternal layer (caruncles) and fetal tissues (cotyledons). Cotyledons begin to form around day 30 of gestation and interdigitate with the caruncles shortly after on day 40 (Diaz et al., 2014). This placentome becomes highly vascularized during pregnancy, and this is the sight of all fetal-maternal nutrient and waste exchange. The proper development and maintenance of placental vascularization is critical for feto-maternal nutrient exchange. Capillaries are detectable by day 18 in fetal membranes (Reynolds and Redmer, 1992; Grazul-Bilska et al., 2011; Bairagi et al., 2016). The vascular area in the gravid uterine horn doubles by day 24 while the non-gravid horn experiences vascular increase around day 30 (Reynolds and Redmer, 1992; Grazul-Bilska et al., 2011; Bairagi et al., 2016). Vascularization of the cotyledon increases due to vessel branching, which increases vascular density, while vascularization of the caruncle increases in individual capillary size (Bairagi et al., 2016). The maternal side of the placenta is comprised of large capillaries that allow for “slow flow”. This “slow flow” is designed for nutrient delivery, while the fetal portion is comprised of highly branched small capillaries that allow for rapid transport of nutrients (Reynolds et al., 2005b). Although the placenta is fully developed by third trimester there is still a dramatic increase in angiogenesis occurring. Data suggest that the endometrium releases angiogenic factors that allow for this increase in capillary size and number (Millaway et al., 1989; Reynolds et al., 1989). During this later portion of gestation blood flow towards the gravid horn increases around 16-fold (Reynolds et al., 2005a). This large increase is due to the

high level of oxygen being transported to the fetus. There are many factors that may affect the vasculogenesis such as animals that experience chronic hypoxia, overnutrition during adolescence, and high levels of nutrient restriction during gestation (Reynolds et al., 2005b). There is some debate on how maternal nutrient availability affects placental angiogenesis. Some describe no effect on uterine blood flow because of maternal nutrient restriction, however a reduction in angiogenic gene expression has been observed (Borowicz et al., 2004; Reynolds et al., 2005b; Luther et al., 2007). This reduction in gene expression may alter placental development and function (Chandler et al., 1985; Leury et al., 1990; Kelly, 1992; Reynolds et al., 2005b). This process of vascularization is a key component to nutrient exchange throughout the entirety of pregnancy. If there is insufficient development of this highly vascularized system, fetal effects may present. Changes to vascularization is most easily observed by a change in placentome number, size, and classification. Placentome formation and function is sensitive to maternal nutritional status and undergoes vascular changes in response to dietary stress (Clarke et al., 1998; Steyn et al., 2001; Osgerby et al., 2004; Vonnahme et al., 2006). The placenta's role in feto-maternal nutrient transfers suggests it is also closely associated with events of developmental programming.

Development of Trophoblast Binucleate Cells

Two cell types emerge from the trophectoderm, mononucleate and binucleate trophoblast cells. BNCs differentiate from trophoblast mononucleate cells beginning on day 14 of gestation through mitotic polyploidy (Seo et al., 2019). BNCs are specific to the ruminant placentas and following conceptus elongation they remain present throughout gestation until the time of parturition (Wooding et al., 1986). Binucleate cell numbers begin to increase following their

differentiation. On day 16, 1% of the trophectoderm is made up of BNC, and by day 18, 15-20% of the trophectoderm consists of BNCs (Wooding and Wathes, 1980; Carnegie et al., 1985; Seo et al., 2019).

Some believe that only mononucleated trophoblast cells are involved in attachment of the conceptus to the endometrium, while BNCs are involved in migration through the endometrium (Spencer et al., 2004). Mononuclear trophoblast cells adhere to the surface of the endometrium while the BNCs migrate through the endometrial layer allowing for the formation of syncytial plaques (Johnson, et. al., 2018). Wooding et al., (1986) described that this cell migration as a function of the developing fetus rather than an initiation by a maternal signal. There is no reduction in the migration rate of BNCs when parturition is initiated or when an abortion occurs (Wooding et al., 1986). Syncytial plaques which are termed “hybrid cells”, form when BNCs migrate into the endometrium and bind to uterine endometrial (UE) cells (Igwebuike, 2009; Klisch and Schraner, 2020). These plaques or hybrid cells are thought to play a role in the efficiency of nutrient transfer across the feto-maternal interface.

The question of what controls the formation and function of BNCs is not well understood. Wooding, et. al (1986) evaluated the change in BNC migration. They performed a hypophysectomy, removed a section of the pituitary stalk, and an adrenalectomy on different subsets of lambs *in utero*. Following these three procedures the percentage of BNCs present in the caruncle did not differ (Wooding et al., 1986). This indicates that developing fetal tissues, such as the pituitary gland, may not influence BNC migration into the uterine endometrium. However, there is cell signaling at play for implantation to occur and this cell to cell signaling may influence the migratory behavior of BNCs. There are many studies that show that maternal

nutritional status plays a direct role in placental function and formation, but there is limited research evaluating how maternal nutrition affects the formation and function of these BNCs. This is an area that requires future research.

Functions of Trophoblast Binucleate Cells

BNCs have various functions that are essential to the establishment and maintenance of pregnancy. These functions include: 1) aiding in the implantation and adhesion of the conceptus to the uterine lining through the formation of syncytial plaques, 2) allowing for efficient diffusion of nutrients across the feto-maternal interface, and 3) the production and release of pregnancy specific hormones such as placental lactogen and pregnancy associated glycoproteins (PAGs) (Wooding, et. al., 1986, Seo, et. al., 2019).

As BNCs migrate through the endometrium, they begin fusing with endometrial cells (Johnson, et. al., 2018). These endometrial cells either remain present after fusion and are absorbed by the BNCs or undergo phagocytosis which is initiated by BNC migration; however there is some debate over the fate of endometrial cells (Wooding, 2022). Syncytial plaques are a specialized hybrid cell which will later form the feto-maternal interface in the placentome (Green, et. al., 2021, Johnson, et. al, 2018). The role and function of these syncytial plaques is not well understood, though they are thought to play a role in the immunological barrier that is the placenta (Wooding, 2022). This immunological barrier limits the risk of bacterial or viral diseases present in maternal circulation from entering the fetal system. An *in-vitro* study performed with placental tissue determined that trophoblast cells present in syncytial plaques contained high levels of major histocompatibility complex type 1 (MHC-1) (Wattegedera et al., 2018). MHC-1 is present on the outer surface of cells that serve as binding sites for maternal

immune cells. This presence of MHC-1 allows for the identification of antigens and can initiate the immune response (Moffett and Loke, 2006; Wattegedera et al., 2018). Pregnancy alters the maternal immune response through placental release of cytokines that promote immune tolerance. There is an upregulation in cytokines associated with inflammation suppression and a down regulation in cytokines that are associated with inflammation (Pastor-Fernandez et al., 2020). These plaques allow the contents BNC granules and immune related cytokines to reach maternal circulation and act as the passage way to maternal circulation (Igwebuike, 2009).

Through the use of serial section counting, ovine syncytial plaques are relatively uniform in shape throughout placentomes and contain roughly 25 nuclei (Wooding, 2022). Ovine uteri develop extensive plaques and remain present throughout the entirety of pregnancy (Green, et. al., 2021). The growth and size of the placenta is determined by the continue division of the trophectoderm, increasing BNC populations and the rate of migration and formation of syncytial plaques (Wooding, 2022). As BNCs differentiate they develop cytoplasmic granules which serve as storage sites for proteins and hormones (Klisch and Schraner, 2020). These cytoplasmic granules contain vesicles, which are later termed exomes when they are released from the cell (Klisch and Schraner, 2020). Vesicles store proteins, lipids, and nucleic acids (Zhang et al., 2019). BNC vesicles contain pregnancy associated glycoproteins (PAGs), placental lactogen, and prolactin related protein-1. Once BNCs invade the endometrium and begin forming syncytial plaques, vesicles are released from the cell allowing for exomes to enter maternal circulation (Klisch and Schraner, 2020). Exomes play a role in cell-to-cell communication. These functions may include local systemic cell modification. This modification could play an important role in the modification of the maternal immune system (Klisch and Schraner, 2020) (insert other

source). Without this modification and down-regulation of the maternal immune response, maternal immune cells would be upregulated in response to the presence of foreign fetal cells.

Placental efficiency is one important factor in the production of healthy offspring. Certain nutrients are required for proper fetal development, such as glucose, amino acids, and fatty acids. These molecules need to be transported from maternal capillaries to fetal capillaries and depending on their size, facilitated diffusion is not possible and there is an energy requirement for transport (Brett et al., 2014). BNCs allow for maternal and fetal circulation to be closer together. Unlike in hemochorial placentas (present in humans and primates) there is no mixing of fetal and maternal blood in ruminant placentas. Without the migration of BNCs, the distance of transport for certain nutrients would be too great, limiting their availability. By shortening the distance for both facilitated diffusion and active transport, there is a decrease in transfer time as well and a decrease in overall energy requirements. The question of how nutrient availability affects the development and function of these BNCs arises. There has been no work evaluating whether BNC function is influenced by maternal nutrient status.

Pregnancy Associated Glycoproteins

PAGs are members of the aspartic proteinase family, which consists of proteolytic enzymes (Beckers et al., 1999). Although they are a part of this enzymatic family, they do not have the ability to act as enzymes because of an alteration in the active site (Beckers et al., 1999; Garbayo et al., 2008). PAGs have been classified into two groups, ancient and modern, based on when and where these PAGs originated (Wallace et al., 2015). Ancient PAGs, as the name implies, have existed far longer than modern PAGs. Using PAG assays the expression of modern PAGs originate from BNCs while the expression of ancient PAGs originates from the remainder

of the trophectoderm (Xie et al., 1994; Beckers et al., 1999; Green et al., 2000; Wooding et al., 2005). PAGs have been used extensively in animal agriculture to diagnose pregnancy and are frequently used in research to assess pregnancy viability (Pohler et al., 2016; Reese et al., 2019) However, the function of PAGs remains unclear. Although PAGs are expressed in cattle and sheep, the timing of expression differs between species. Ovine PAG (oPAG) 2 is expressed from mononucleated trophoblast cells and is classified as ancient, while oPAGs – 1, 3, 4, 5, 6, 7, 8, and 9 are expressed from BNCs and classified as modern (Green et al., 2000). The expression of bovine PAG (bPAG) – 1 is classified as modern, whereas bPAG – 2, 8, 10, 11, 12, and 13 are classified as ancient (Xie et al., 1994; Green et al., 2000; Wooding et al., 2005).

The presence and function of PAGs in maternal circulation has been of interest to researchers. As BNCs migrate into the endometrium, they release the contents of their vesicles, as previously discussed. This release is how PAGs, and other pregnancy associated proteins and hormones reach maternal circulation and are detectable via blood sampling. The time periods in which PAGs are detectable in circulation coincide with landmark events of embryonic development. By day 28 in cattle, implantation has occurred and placentation has begun. During this process, BNCs migrate into the endometrium, forming syncytial plaques, and release PAGs and pregnancy related hormones into the maternal system. This is when either low or “normal” concentrations of PAGs are detectable. Evidence suggests there is an association between concentrations of PAGs and BNC function and placental health (Pohler et al., 2013a; Reese et al., 2019). This association can give producers and scientists an opportunity to assess pregnancy viability early on and throughout gestation.

PAGs are detectable via enzyme-linked immunosorbent assay (ELISA) in maternal blood and their concentrations vary depending on day of gestation. ELISAs are used frequently in cattle to diagnose pregnancy and embryonic loss, while more recently researchers have begun using them in sheep (Pohler et al., 2013a; Pohler et al., 2016; de Miranda et al., 2017; Reese et al., 2019). The exact date of expression and when PAGs are first detectable has varied. In cattle PAGs are first detectable between days 24 and 26 of gestation (Green et al., 2005; Pohler et al., 2013a). However, in sheep there is some evidence that oPAG-2 can be detected as early as day 13 whereas others have found though the use of serum and ELISA, they are not detectable until day 30 of gestation (Green et al., 2000; de Miranda et al., 2017). The use of ELISAs is as reliable of a source of pregnancy diagnosis as the use of ultrasonography (de Miranda et al., 2017). Blood collection requires less training and upfront costs for producers. PAGs are also used extensively in the cattle industry to detect early embryonic loss. Early detection of embryonic loss allows producers to make more informed management decisions earlier in the breeding season. Detecting early embryonic loss prevent the risk of increasing the calving interval and allows producers to cull females that are unable to maintain a pregnancy. Embryonic loss is categorized as early or late. If it occurs prior to day 28 of gestation it is early, and after day 28 it is late (Pohler et al., 2016). If PAG levels are increased as day 30 of gestation approaches there is lower probability of embryonic loss, and animals with higher circulating levels of PAGs between days 28 and 100 of pregnancy are more likely to maintain a pregnancy (Pohler et al., 2016; Gatea et al., 2018). A similar study where high fertility and sub-fertile heifers were compared reported similar results (Reese et al., 2019). Both high fertility and sub-fertile heifers that experienced early pregnancy loss had low levels of circulating PAGs by day 28 compared to those who

maintained their pregnancy (Reese et al., 2019). The high fertility animals that experienced pregnancy loss by day 44 of gestation had lower levels of PAGs when measured on day 28 of pregnancy compared to the sub-fertile group (Reese et al., 2019). Interestingly, the group of sub-fertile heifers that experienced similar pregnancy loss by day 44, did not show a change in PAG concentrations (Reese et al., 2019). These researchers defined fertility status based on pregnancy success. Authors noted that the chances of pregnancy maintenance after day 28 increase by 7.2% for every 1 ng/mL rise in PAG concentrations, but if PAG concentrations were to decrease by 1ng/mL during this time there is a 20% increase in risk of pregnancy loss (Reese et al., 2019). In beef cattle, circulating concentrations of PAGs less than .72 ng/mL by day 28 of gestation result in a 95% chance that the pregnancy will be lost (Pohler et al., 2016). Levels of PAGs fluctuate as pregnancy progresses. Concentrations, slowly increase during early gestation, plateau, and then rapidly rise as parturition approaches (Green et al., 2005; Pohler et al., 2013a; Ricci et al., 2015).

In cattle, the administration of PAGs to luteal cells has resulted in increased prostaglandin E2 (PGE2), suggesting that PAGs have luteotrophic action (Del Vecchio et al., 1995; Weems et al., 1998; Egen et al., 2009; Wallace et al., 2015). This increase in PGE2 leads to an increase in progesterone from luteal cells on the ovary. PAGs have also been associated with the downregulation of the maternal immune system (Dosogne et al., 2002). *In vitro* PAG-1 can be immunosuppressive (Hoeben et al., 1999). At the time of lambing PAG levels peak while cells associated with immune response are low (Egen et al., 2009). The functions of PAGs still needs to be investigated because of varied results when studied *in vitro* compared to *in vivo* models. However, pregnancy rates in ewes vaccinated against PAGs were not affected indicating PAGs are not needed for the establishment and maintenance of pregnancy (Egen et al., 2009).

Developmental Programming

When insults are applied during critical periods of fetal development, the function and structure of fetal cells, organs, and organ systems are affected (Ford and Long, 2011; Piaggio et al., 2018). These insults can be defined as environmental (altitude, handling stress, harsh weather conditions, etc.), maternal plane of nutrition (energy, protein, mineral supplementation, or restriction, etc.), and the risk of contracting disease or ingestion of toxins. Exposure to varying levels of insults during fetal development will affect an animal pre and postnatally, and when that animal enters the production system. Effects in offspring due to a maternally applied insult is referred to as “fetal programming” or “developmental programming”. Maternal dietary insults are commonly experienced by Montana livestock due to frequent drought conditions previously described. Maternal nutritional status has a long-term effect on fetal growth and health later in life (Reynolds et al., 2010; Caton et al., 2019a). Both over- and under-nutrition can cause fetal programming effects.

The first and second trimesters account for about 100 days of pregnancy. Following implantation and establishment of pregnancy the first two trimesters are dedicated to placental development. Implantation of the conceptus to the entirety of the endometrium is complete around day 22 (Stenhouse et al., 2022). After implantation, BNCs begin migration into the endometrium, and embryonic membranes, such as the allantois and chorioallantois begin to form and increase in size. During this time, the cotyledons begin to form and vascularization of the placentome increases. This increase in vascularization allows for nutrient and waste exchange. Only 10% of fetal development occurs in the first 90 days of gestation, by this time the placenta has reached its maximum weight (Redmer et al., 2004).

First and second trimester dietary insults and developmental programming effects

Early gestation is a time of vast cellular remodeling as the embryo will transition from a 2-cell structure to a fetus containing highly specialized cell types and organs structures. During first trimester, primary muscle stem cells develop and primary myogenesis occurs. Reports indicate that when 50% restriction of total digestible nutrients (TDN) is applied during early gestation, between days 28 and 78, signals for fetal muscle development are downregulated and offspring experience a reduction in the ratio of primary to secondary myofibers (Zhu et al., 2004). Gauvin et al. (2020) performed a similar study and restricted ewes to 60% TDN with similar results. Samples from this study were collected on day 90 of gestation and the restricted group had a decreased ratio of primary to secondary muscle fibers (Gauvin et al., 2020). Secondary myofibers differentiate from primary myofibers, therefore, a reduction is this ratio will have long term effects on muscle hyperplasia. All muscle fibers an animal will have throughout its life develop *in utero*, however as an animal ages, these muscle fibers undergo hypertrophy, increasing in size (McCoski et al., 2021). This reduction in muscle cell formation because of early maternal restriction has a negative impact on offspring productivity and performance (Zhu et al., 2004).

Organogenesis begins during early to mid-gestation and is complete during third trimester. Nutrient restriction during this critical period leads to decreased organ weights in lambs (Vonnahme et al., 2003; Field et al., 2015). When ewes were fed 50% of TDN beginning on day 28 of gestation and continuing until day 135, a reduction in fetal weights and lamb hearts, lungs, liver, spleen, and kidneys were seen in offspring from restricted mothers (Sandoval et al., 2021). A similar study yielded comparable results, (Vonnahme, et al., 2003). In this study ewes

were fed 50% of TDN between day 28 and 78. Resulting lambs had lighter livers, lungs, and kidneys at day 78 of gestation (Vonnahme et al., 2003). This difference in organ weight can predispose offspring to coronary heart disease and hypertension (Barker and Clark, 1997). Abnormal organ development may lead to reduced offspring vigor as organs are unable to function in a manner to support offspring health.

The effects of maternal restriction during early to mid-gestation on the reproductive health of offspring is a topic not well understood. Many studies on the health and productivity of offspring that experienced nutrient restriction while *in utero* do not take offspring sex into account. Male fetuses on average are bigger than females during the first and second trimesters and may be because male embryos develop much quicker than their counterparts (Xu et al., 1992; Alur, 2019). In fetal lambs, the differentiation of gonads can be detected on day 31 of gestation (Zamboni et al., 1979). While it seems likely that dietary insults during this time will affect gonad development and function, the application of dietary insults during early to mid-gestation have yielded different results. In one trial, ewes were fed to 100% or 50% of metabolizable energy (Rae et al., 2002). Authors report no difference in scrotal circumference between ram lambs of the two treatments; however, ewe lambs from restricted ewes had lower ovulation rates (Rae et al., 2002). Although there were no differences in ram lambs, early gestation is when seminiferous tubule development occurs and when Sertoli and Leydig cells begin to differentiate (Hochereau-de Reviers et al., 1987). The effects on both prenatal ovary and testicular development are dependent on the time nutritional insult is applied. At term, ram lambs born from ewes that were restricted by 50% from day 31 – 100 had fewer Sertoli cells per testicle and lighter paired testicle weights than their control counterparts (Bielli et al., 2002). These

effects to both male and female reproductive organs give cause for concern for future reproductive health and efficiency of breeding animals. Lower ovulation rates and Sertoli cell numbers are indicative of decreased fertility. This decreased fertility limits the number of offspring an animal will produce throughout its life, but also shortens the time they are in the flock. Replacement ewes and rams are selected for their ability to produce healthy offspring each breeding season and to continue to do that for several consecutive breeding seasons. Lack of reproductive success causes sheep producers to cull animals each year, ultimately limiting their breeding herd and number of offspring. Collectively, although nutrient requirements are not as high during early to mid – gestation, restriction can still lead to offspring with poor health and poor reproductive performance.

Third trimester dietary insults and developmental programming effects

The third trimester begins around day 100 of gestation in sheep. During the final 50 days, about 90% of fetal growth occurs (Redmer et al., 2004). Maternal nutrient requirements increase between 30-50% during this time (Caton et al., 2019b). The increase in energy requirements is a result of fetal growth. Half of the available nutrients are partitioned to support gravid uterine tissue and vascularization, 25% is needed to support fetal growth, and 25% is needed to support normal maternal metabolic functions (Caton et al., 2019b). Maternal dietary restriction during this time frequently results in smaller fetal birth weights.

When mothers are restricted only during the latter portion of gestation, a decrease in fetal births weights is seen as a result, though with varying severity. Ewes that were restricted by 55% of the maintenance diet during the third trimester had lambs with similar birth weights as those from the control group (Faichney and White, 1987). Similar studies yielded differing results.

When underfeeding occurred to achieve a predetermined level of blood glucose (1.0 – 1.4 mmol/l of blood) from day 112 until term, their lambs had lighter birth weights compared to the control group (Mellor, 1983). Lighter weights at birth can lengthen the period it takes for a newborn to stand and receive colostrum, weakening their immune system and making them susceptible to disease (Miller et al., 2010; Pettigrew et al., 2021). Lighter birth weights also set individuals at a disadvantage compared to their heavier counterparts. Those counterparts tend to be heavier at weaning and have a higher rate of gain throughout their lives (Miller et al., 2010). For producers, pounds of gain equate to dollars in revenue.

It is well known by scientists and producers that dam nutrient availability can lead to negative fetal affects. However, the results are inconsistent. This may be due to the timing of restriction during gestation, parity of the dam, pre-existing conditions, living environment, and sex of the offspring. These factors pave the way for future developmental programming work.

Placental Response to Nutritional Stress

The placenta is a dynamic organ, and as it develops through the earlier part of gestation, it undergoes some compensatory mechanism to mitigate nutrient restriction. The ability of the placenta to compensate for nutrient availability is dependent on what nutrients are restricted, the severity of the restriction, and the timing of restriction. Maternal body condition score, health status, disease status, and living environment are also involved in the ability of placenta to overcome nutrient restriction. Placental adaptation can be evaluated by observing placentome development, angiogenic related genes, uterine and placental vascularization, and fetal birth weights. Early pregnancy is when a majority of the placenta and placentomes are formed.

Placental alterations are observed if nutritional insults are applied within the first 90-days (Steyn et al., 2001; Vonnahme et al., 2011; Reynolds et al., 2013).

Angiogenesis and placental vascularization begin around day 16 and capillaries steadily increase in number and size as pregnancy progresses (Grazul-Bilska et al., 2010; Grazul-Bilska et al., 2011). Placental vasculogenesis is highly regulated by angiogenic factors such as placental growth factor (PGF), vascular endothelial growth factor (VEGF), angiopoietins (ANG1/2), etc. This process appears to be sensitive to maternal nutritional status. Data indicate the nutrient restriction during mid gestation decreased mRNA transcription of *VEGF* in the ovine model (McMullen et al., 2005). VEGF is produced by cells to stimulate the formation of new blood vessels. Late term nutrient restriction inhibits fetal growth. Lambs from this study had lighter birth weight compared to those that came from adequately fed mothers, likely due to altered nutrient transfer resulting from abnormal angiogenesis (McMullen et al., 2005).

Placentomes are responsive to maternal nutritional status, and undergo morphological changes to regulate transfer to the developing fetus (Vonnahme et al., 2008). A visual placentome classifications system was developed to identify morphological changes that may occur (Vatnick et al., 1991b). They are categorized into four groups, A, B, C, or D based on the amount of cotyledonary tissue present. Type A cotyledons contain the least amount of cotyledonary tissue, while type D contains the most amount of cotlydeonary tissue (Vonnahme et al., 2006). There have been varying results if maternal plane of nutrition affects placentome type, placental weight, and cotyledon weight. When maternal nutrient restriction is applied during this first 90 days, a reduction in total cotyledon weight and total cotyledon number has been seen, but no change in total placental weight (Clarke et al., 1998; Osgerby et al., 2004; Ma et al., 2011).

However, ewes adapted to lifestyles of prolonged restriction (grazing rangelands) have higher number of type D placentomes (Vonnahme et al., 2006). This indicates that type D placentomes develop as a compensatory mechanism by increasing the area of fetal attachment. Increased fetal attachment area, increases the amount of surface area that exchange nutrients and waste. The study by Clarke et al, (1998) did not record a difference in the number of each classification of placentome; however, a similar study performed by Heasman et al (1998) observed a difference in the type of placentome present. In this study, ewes were provided with half of their maintenance energy requirements. Authors observed a greater total number of placentomes in the restricted group, but the restricted group had more type B-D placentomes, indicating more fetal tissue surrounding maternal tissue (Heasman et al., 1998). The transition to differing placentome types can even be observed when animals are exposed to a shorter duration of restriction and are then realigned to maintenance diets. Ewes were exposed to 50% restriction from day 28 to 78 of gestation and then realimented to maintenance (Ma et al., 2011). At lambing there was no difference in placentome weight or total number of placentomes, but there was a higher number of type D placentomes in the restricted/realimented group compared to the control group (Ma et al., 2011). This shift towards type D placentomes indicates that there is a placental response to lack of nutrients. This increase in fetal tissue leads to an increase in fetal vasculature and an increase in fetal nutrient uptake.

Nutrient availability affects diffusion and transport of nutrients to the fetus. Minimal levels of nutrient restriction can promote placenta growth or retard it (Kelly, 1992; Robinson et al., 1994; Clarke et al., 1998). This may be due to fetal signaling to upregulate growth therefore increasing nutrient transfer (Diaz et al., 2014). Research in mice provided evidence that when

there is stress due to low nutrient availability, fetuses signal for an increase in placental growth, in turn increasing the vascular network (Constância et al., 2005; Sferruzzi-Perri et al., 2013). Studies in rats limiting protein in the diet decreased placental and fetal weights (Malandro et al., 1996; Belkacemi et al., 2010). Glucose is an important nutrient for fetal development, especially during third trimester when most of fetal growth is occurring and when maternal requirements are highest. All glucose required by the fetus must come from the maternal system and is transported across the placenta through glucose transporters (GLUT). Studies done when dietary restriction is applied have seen placentas from restricted animals are lighter but have an upregulation in the number of GLUT-1 receptors (Dandrea et al., 2001). Similar results were seen when ewes were restricted by 50% of dietary requirements during the first half of gestation and then alimented to maintenance until term. The restricted group had higher *GLUT-1* and *3* transcription both while the restricted diet was being applied (day 78) and at term (Ma et al., 2011). This increase in the expression of *GLUT* indicates a placental response to increase the transport of glucose into fetal circulation.

There has not been extensive work evaluating the influence that maternal nutritional status and other stressors have on concentrations of PAGs. Low levels of PAGs may indicated poor placental health, and research has seen that undernutrition does influence circulating hormones (Chelikani et al., 2009; Steinhauser et al., 2021). PAGs, progesterone, and placental lactogen (PL) are factors expressed solely by the placenta and placental related cells. They can be used as a tool to assess placental health and pregnancy viability. These factors are measurable by producers and give them insight into the health of pregnancies during periods of scarce feed or disease.

Current research shows that ewes experiencing nutritional insult through over feeding have expressed PAG 4 and 9 while these PAGs were not expressed in a group of lean/control ewes (McCoski et al., 2018). PAG 4 and 9 are expressed solely by BNCs giving some indication that these BNCs may be sensitive to maternal nutritional status, and that PAGs could be a tool to assess the health and function of BNCs and the placenta. Although researchers are unsure of whether maternal stress and nutrient status can be determined by evaluated PAGs, it is well known that PAGs are successful tool in pregnancy detection. The use of this method of pregnancy diagnosis is commonly used in the dairy industry, where embryonic loss is a common occurrence.

Progesterone is a key hormone in the maintenance of pregnancy. Following day 50 of gestation, the corpus luteum is no longer needed, and the placenta can produce adequate progesterone levels for pregnancy maintenance (Al-Gubory et al., 1999). Progesterone levels in maternal circulation can be used as a tool to determine placental health and efficiency. When progesterone levels in maternal blood supply were compared on day 78 of gestation between a group of control ewes (fed to 100% NRC requirements) and a restricted group of ewes (fed to 50% of requirements), progesterone levels did not vary (Vonnahme et al., 2003). However, a similarly performed study yielded different results. Lemley, et. al 2018 fed gestating ewes either 100% or 60% of NRC requirements and began diets on day 50 of gestation. Blood from the maternal saphenous artery (located on the medial side of the pelvic limb), uterine vein of the gravid horn, and the umbilical artery and vein were collected for progesterone measurements (Lemley et al., 2018). Progesterone levels (ng/mL) were higher in the uterine vein of the restricted ewes but there was no difference in progesterone levels in the saphenous artery or

umbilical vessels (Lemley et al., 2018). Other researchers fed ewes a diet for high rate of gain or moderate rate of gain and collected blood three times a week for the entirety of pregnancy to evaluate progesterone levels (Lea et al., 2007). There was no difference in progesterone levels between dietary groups in the first trimester but during the second and third trimesters of pregnancy, the high rate of gain animals had lower mean levels of progesterone compared to the moderately fed group (Lea et al., 2007). This study also consisted of ewes that were fed the diets described above and were slaughtered at either day 81 or 130 of gestation. On day 130, the placentas from the high rate of gain group were lighter than those from the moderate group. These ewes also had lower levels of circulating progesterone (Lea et al., 2007). This lower level of progesterone in the heavy group may be due to the increase in the hepatic clearance.

Placental efficiency can also be measured by evaluating levels of placental lactogen (PL) in maternal circulation. PL originates from the same gene family as prolactin and growth hormone (Anthony et al., 1995). Being that PL and prolactin are similar in structure, PL can bind and react with prolactin receptors in the uterus. PL is released by BNCs upon migration into the endometrium and levels in maternal circulation can be used to assess BNC migratory behavior (Regnault et al., 1999). Levels of PL are dependent on the stage on gestation, as pregnancy progresses PL in maternal circulation begins to increase and remains high throughout third trimester (Byatt et al., 1992a). PL is also involved in mammogenesis and is commonly associated with milk production in dairy cattle (Riddle et al., 1933; Lacasse et al., 2016). There is some indication that PL is involved in the regulation of fetal growth through the manipulation of maternal metabolism (Byatt et al., 1992b; Oliver et al., 1992). In ewes, prolactin is involved in the regulation of epithelial gland function and prolactin and progesterone act on one another to

increase concentrations (Chilton et al., 1988; Cassy et al., 1999). Work in ewes fed a high or moderate rate of gain throughout pregnancy to evaluate PL levels, observed that animals fed a high rate of gain had lower levels of PL beginning around day 50 (GLUCKMAN et al., 1979; Kappes et al., 1992; Lea et al., 2007). PL is first detectable around day 40 to 50 in ovine pregnancies (GLUCKMAN et al., 1979; Kappes et al., 1992). The direct role that PL has in fetal development is still a popular topic for researchers. Leibovich, et. al (2000) immunized ewes against PL and evaluated lamb birth weights. The immunized group had heavier lambs than their control counterparts. One would think that if PL is involved in the regulation of fetal growth and metabolism, that lambs born of immunized ewes would be lighter than their counterparts. However, similar studies where animals were immunized against growth hormone (which is in the same family of hormones as PL) improved growth aspects (Bomford and Aston, 1990). The immunization of growth-related hormones resulting in enhanced activity is not well understood. A hypothesis behind this is that immunization of PL triggers a feedback mechanism in the placenta to increase PL production, which in turn would alter placental formation and function (Leibovich et al., 2000). Although, PL originates from BNCs in the ruminant placenta, the use of PL as a direct measurement of BNC and placental function has yet to be tied together.

Conclusion and Implications

Although the placenta is a temporary organ it plays an essential role the longevity and health of offspring. Optimizing placenta efficiency, specifically BNC health, may not be at the forefront of producers' minds. However, the use of PAGs and other pregnancy related - hormones gives producers insight into how to adjust their management strategies to produce a

better lamb crop. This assessment can also shed light into the timing of certain management strategies, such as when to increase feed availability or offer some form of supplementation.

There are many factors that play a role in the development of healthy offspring, and it is well known that the placenta plays a large role. Although there is a large amount of research pertaining to the placenta's role in nutrient transfer and optimizing fetal growth despite certain stressors, the role BNCs play in this nutrient transfer is lacking. The question of whether BNC function and migration is sensitive to maternal nutrient availability remains unanswered.

CHAPTER THREE

EWE UNDERNUTRITON ALTERS COTYLEDON DEVEOPMENT AND FUNCTION

Introduction

As placentation in ruminant species occurs, the uterine endometrium is partially eroded away by BNCs, classifying ruminant placentas as synepitheliochorial (Grigsby, 2016). This migration and partial erosion by BNCs aids in efficient nutrient transfer (Wooding and Wathes, 1980). Nutrient transfer occurs at specialized areas across the placenta, referred to as placentomes. Placentomes have the ability to change their shape in response to nutrient availability (Heasman et al., 1999; Steyn et al., 2001; Vonnahme et al., 2006; Vonnahme et al., 2011). It has been observed that when gestating ewes experience nutrient restriction, placentomes increase the amount of fetal tissue, most likely to increase surface area for greater nutrient exchange (Reynolds et al., 2005b). Trophoblast binucleate cells release pregnancy specific hormones and proteins, in turn making them detectable in maternal circulation (Wooding, 1984). These pregnancy specific proteins, PAGs, are often used as a management tool by producers to diagnose pregnancy and to assess pregnancy viability (Pohler et al., 2016). Low levels of PAGs in cattle have been associated with early embryonic loss (Reese et al., 2019). Pregnancy associated glycoproteins that are expressed by BNCs may be a way to assess placental and cell health during periods of dietary stress.

Nutrient restriction is a common occurrence in sheep raised on rangelands. Ewes raised on rangelands are bred later in the year which coincides with the decline in forage amount and quality and these ewes can experience chronic nutrient restriction, leading to fetal programming

effects. Although changes to placentome shape have been observed in response to dietary stress, the effect of dietary restriction on BNC behavior and function has not been evaluated. Therefore, it is hypothesized that cotyledons are sensitive to chronic nutrient restriction leading to changes in BNC behavior and PAG concentrations. The main objectives of this study were to 1) identify biological pathways within cotyledons that were altered by nutrient restriction and 2) assess physical characteristics of cotyledons (such as weight, number, and size).

Materials and Methods

Animals

All animal work was completed in accordance with Montana State University Agricultural Animal Care and Use Committee (AACUC) protocol number 221-AA08.

Prior to the start of the trail, 34 multiparous Targhee ewes, ages 4.5 ± 2 years, were estrus synchronized using a controlled internal drug release (CIDR) device. CIDRs were inserted vaginally and remained in place for 10 days. Following the removal of the CIDRs, ewes were bred to two Targhee rams. These rams were removed after 10 days. Four ewes were bred by the cleanup rams and were excluded from the study. Twenty-four days following ram exposure, ewes were allocated into two dietary treatments with two pens/treatment, 8 animals in each pen. Animals were grouped based on age and starting body weight. Control (CON) animals were fed to 100% of NRC requirements and the nutrient restricted (NR) animals were fed to 60% of NRC requirements (Council, 1985) (Table 1). Ewes were fed once daily through lambing. Diets were adjusted according to the trimester of pregnancy to ensure control diets were meeting ewe gestational requirements. Water, loose mineral, and salt were offered *ad libitum.* Composition of

the loose mineral included minimum calcium 11%, phosphorus 7.9%, selenium 35.9%, vitamin A 249,400 IU/lb, vitamin D 24,900 IU/lb, and vitamin E 500 IU/lb.

Pregnancy was diagnosed via transabdominal ultrasound on day 70 of gestation and open ewes (n = 4) were removed from the trial. Body weight (BW) and body condition scores (BCS) were recorded every 2 weeks until lambing. At lambing, each ewe was moved to a lambing jug with *ab libitum* alfalfa and water until 24 hours post lambing. Ewes and lambs were then reintroduced into the flock and returned to maintenance diets. A diagram outlining the timeline can be found in Figure 1.

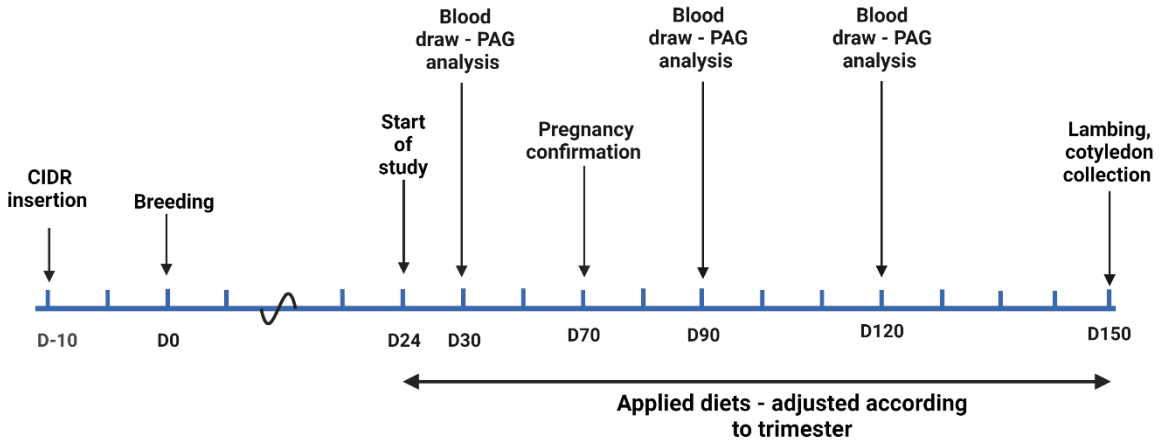

Figure 1: Study timeline

Table 1: Nutrient composition of hay fed, amount of each nutrient fed to each ewe according to trimester of gestation.

Item	Hay (%)	First trimester		Second trimester		Third trimester	
		CON	NR	CON	NR	CON	NR
Dry Matter intake, kg/day		1.4	0.8	1.7	1.02	2.3	1.38
Dry matter	89.7	1.26	0.72	1.52	0.91	2.06	1.24
crude protein	17.4	0.22	0.12	0.27	0.16	0.36	0.22
ADF	31.1	0.07	0.04	0.08	0.05	0.11	0.07
NDF	37.2	0.03	0.01	0.03	0.02	0.04	0.02
TDN	63	0.02	0.01	0.02	0.01	0.03	0.02
Ash	9.87	0.00	0.00	0.00	0.00	0.00	0.00
Ca	1.35	0.00	0.00	0.00	0.00	0.00	0.00
Phosphorus	0.26	0.00	0.00	0.00	0.00	0.00	0.00
Magnesium	0.22	0.00	0.00	0.00	0.00	0.00	0.00
Potassium	2.55	0.00	0.00	0.00	0.00	0.00	0.00
NEm	1.4	0.00	0.00	0.00	0.00	0.00	0.00
NEg	0.82	0.00	0.00	0.00	0.00	0.00	0.00

Blood collections and analysis

Blood was collected via jugular venipuncture on days 30, 90, and 120 of pregnancy. After collection, BD Vacutainer tubes with K2EDTA anticoagulant, were kept on ice until plasma could be isolated. Samples were centrifuged at 1,500 x g for 15 minutes at 4 °C. After spinning in the centrifuge, plasma was removed and transferred to 2 mL microcentrifuge tubes. The buffy coat and red blood cells were discarded. Samples remained in the -20 °C freezer until PAG concentrations were measured by in-house ELISA (Pohler et al., 2013b).

Placental sample collection

Immediately following the expulsion of the placenta, total placental weights, and total number of cotyledons per placenta were recorded.

Cotyledons (n = 6 / placenta) were collected for RNA sequencing and tissue staining. Cotyledons were selected from multiple locations on the placenta to achieve a representative sample. Following the collection, the weight and diameter of each cotyledon was recorded. Cotyledons collected for RNA sequencing were stored in RNAlater (thermoFisher). Samples were stored at -80°C until RNA was extracted. Samples selected for tissue staining were fixed in 4% paraformaldehyde (PAF) and remained at room temperature until processing.

Cotyledon processing for RNA sequencing

Cotyledon samples were removed from the -80ºC freezer and thawed on ice. A sample was then removed from each cotyledon (n = 3) for processing. The remainder of the cotyledon was re-frozen at -80ºC. Samples were placed in a sterile 50 mL conical tube with 500 µL of lysis buffer from the (thermoFisher, PureLink RNA mini kit) and then homogenized. The homogenizer was cleaned between each animal to avoid contamination. The cleaning process consisted of running homogenizer in 45 mL of nanopure water, 45 mL of 70% ethanol, 45 mL of RNaseaway, and 45 mL of nanopure water. Following homogenization, samples were centrifuged at 2,600 x g for 5 minutes at room temperature. The supernatant was then transferred to a 15 mL vial. Equal parts of 70% ethanol were added to the supernatant of each sample, while the solid portion was discarded. The liquid samples were then vortexed briefly and used for RNA isolation following manufacturer's instructions (thermoFisher, PureLink RNA mini kit).

The concentrations and quality of each RNA sample were evaluated on a NanoDrop spectrophotometer (NanoDrop One, ThermoFisher Scientific, Pittsburgh, PA). The NanoDrop was calibrated using 1 µL of RNA free water. Samples having a A260/A280 value between 1.8 and 2.0 and a 260/230 between 1.8 and 2.1 were deemed adequate for RNA sequencing.

Following RNA extraction, samples were kept at -80ºC. Extracted RNA was sent to Novagene (Sacramento, CA) for sequencing and bioinformatics (n = 3 placentas per treatment group) (n = 6 placentas for made comparisons). At Novagene the mRNA was purified using poly-T oligo-attached magnetic beads and fragmentation was performed under elevated temperatures in First Strand Synthesis Reaction Buffer (5x).

Quality control

The raw data was processed through in-house perl scripts and clean data was collected. This clean data was collected by removing reads that contained adapter, ploy-N, and anything low quality. The Q20, Q30, and percentage of guanine – cytosine (GC) was calculated for all clean data. All analyses performed afterwards used the clean data.

Reference genome

Reference genome is ensembl_ovis_aries_oar_v3_1_gca_000298735_1. The index for the reference genome was built using Hista 2 v2.05 and the paired-end clean reads aligned to the reference genome using Hisa 2 v2.05.

Novel transcripts

StringTie (v1.3.3b) was used to assemble the mapped reads for each sample. StringTie uses a network flow algorithm and an assembly step for full length transcripts that represent multiple splice variants for gene locus.

Quantifying gene expression level

FeatureCounts v1.5.0-p3 was used to count the number of reads mapped to each gene. The fragments per kilobase of transcript sequence per millions (FPKM) for each gene was

calculated based on gene length and the number of read mapped. FPMK considers effects of sequencing depth and gene length for read counts.

Differential expression analysis

The comparison between treatment groups based on the number of fetuses was performed using DESeq2 R package (1.20.0) (Love, et. al 2013). The model to determine differential expression was based on a negative binomial distribution. *P-values* were adjusted using the Benjamini - Hochberg's approach for controlling for false discovery rate. Genes with an adjusted *P-value* of < 0.09 were considered differentially expressed.

Read counts for each sequenced library were adjusted by edgeR program package through a normalized scaling factor (Robinson, et. al 2010, McCarthy, et. al 2012). Differential expression analysis of two conditions was performed using the edgeR package (3.22.5) (Robinson, et. al 2010, McCarthy, et. al 2012). These P-values were also adjusted with the Benjamini and Hochberg method. Corrected *P-values* of 0.09 and fold change of two were set as the threshold for significance.

GO and KEGG analysis of differentially expressed genes

GO analysis of differentially expressed genes was implemented by the clusterProfiler R package. GO terms with corrected *P-value* < 0.05 were considered significantly enriched by differential expressed genes. The KEGG database for understanding high level function and use of biological systems. The clusterProfiler R package was used to test the statistical enrichment of DEGS in KEGG pathways.

Statistical analysis

The MIXED procedure in SAS version 9.4 was used for quantitative data. Comparisons between ewe BW, placental weight, lamb birth weight, cotyledon number and average weight were made between treatment groups. Least square means was used to evaluate ewe body weight to determine a treatment by time interaction. Least square means was also used to evaluate a treatment by time interaction for lamb birth weight and placental parameters. Significance for continuous data was set to be $P < 0.05$.

Results

Ewe and lamb data

There was no effect of diet on ewe body weight until day 72 of gestation. CON ewes were heavier on days 72, 86, 100, 114, 128, and 142 of gestation than those in the NR group ($P < 0.05$; Figure 2). Ewe diet did not affect lamb birth weight or the frequency of twins of singletons ($P > 0.1$).

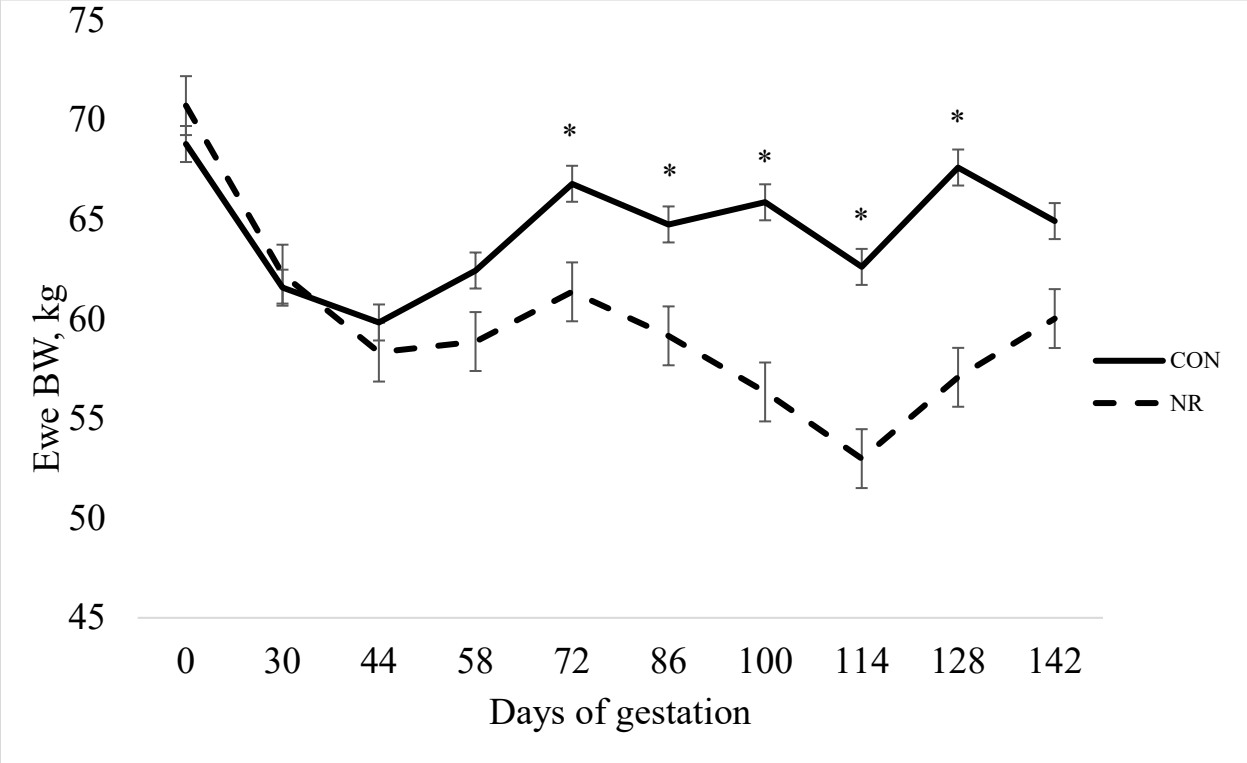

Figure 2: Ewe body weights throughout gestation. Average body weight change of NR and CON animals. * denotes time x treatment interaction; $P < 0.05$.

Cotyledon and placenta phenotypic data

Ewe diet had no effect on cotyledon diameter or cotyledon number ($P < 0.775$). However, cotyledons from CON ewes were an average of 0.55 g heavier than those from the NR group (2.6919 ± 0.009 and (2.139 ± 0.009), respectively; ($P < 0.001$). There was no difference in total placental weight due to dietary treatment ($P > 0.1$).

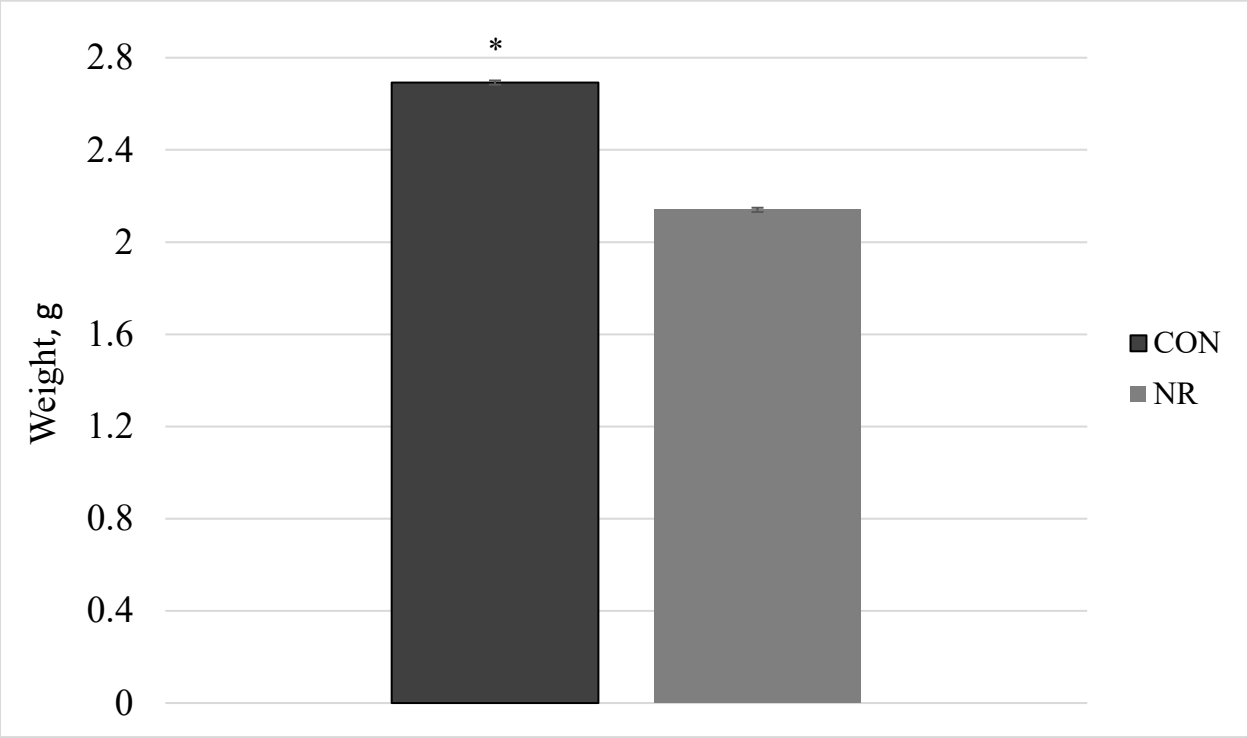

Figure 3: Average cotyledon weight based on treatment group. * denotes an effect of treatment; $P < 0.05$.

RNA sequencing

Data quality

Sequencing averaged 47,261,193 raw reads per sample. An average of 83.41% of reads mapped to the ovine reference genome. Data quality was assigned using Q-scores. The average Q30 score across samples was 92.59%, and G-C content averaged 52.82% (Table 2).

Table 2: Quantification and quality of mRNA reads

Sample	*Raw Reads*	*Raw Bases*	*Clean Reads*	*Clean Bases*	*Error (%)*	*Q20 (%)*	*Q30 (%)*	*GC Content (%)*
T6040	63,364,426	9.5G	61,764,760	9.26G	0.03	97.42	93.06	52.15
T8060	45,526,074	6.83G	44,248,152	6.64G	0.03	97.14	92.57	53.66
T8110	47,679,164	7.15G	46,233,804	6.94G	0.03	97	92.32	53.36
T9594	47,419,926	7.11G	46,171,960	6.93G	0.03	97.28	92.86	52.38
T9634	45,024,058	6.75G	43,974,272	6.6G	0.03	97.27	92.9	52.84
T6373	45,267,928	6.79G	44,168,102	6.63G	0.03	97.39	93.07	53.38
T7056	39,495,108	5.92G	38,266,976	5.74G	0.03	97.27	92.87	51.46
T8035	47,867,378	7.18G	46,063,658	6.91G	0.03	97.07	92.54	51.81
T9430	47,135,460	7.07G	45,926,882	6.89G	0.03	97.05	92.53	53.59
T9537	46,269,408	6.94G	45,096,490	6.76G	0.03	97.12	92.55	52.93
T8123	48,765,490	7.31G	47,184,724	7.08G	0.03	96.53	91.47	52.77
T8135	4,331,990	6.5G	42,182,576	6.33G	0.03	97.04	92.36	53.51

Raw Reads: read count from the raw data.

Raw Bases: base number of raw data, (number of raw reads)*(sequence length), converted to unit (G).

Clean Reads: read count filtered from raw data, used for all downstream analysis.

Clean Bases: base number of raw data after filtering, (number of clean reads)*(sequence length), converted to unit (G).

Error: base error rate of whole sequencing.

Q20: percentage of bases whose Phred values were & gt; 20. Meaning the call error rate is predicted to be 1/100 bases, leading to an accuracy rate of 99% for total bases.

Q30: percentage of bases whose Phred values were & gt; 30. Meaning the call error rate is predicted to be 1/1000 bases, leading to an accuracy rate of 99.9% for total bases.

GC Content: percentage of nucleotides in the read identified as Cytosine and Guanine

Differentially expressed genes

Differential gene expression analysis comparing cotyledons from NR singletons vs the CON singletons identified 470 differently expressed genes (DEGs; $P < 0.05$; 187 up-regulated, 283 down-regulated, Figure 4). The Benjamini – Hochberg adjustment revealed 3 DEGs, cytochrome P450 aromatase, poly [ADP-ribose] polymerase, and one novel gene ($Padj < 0.05$). A comparison of cotyledons from the NR twins vs CON twins identified 426 DEGs ($P < 0.05$; 195 up-regulated, 231 down-regulated). Benjamini – Hochberg adjustment revealed there were no DEGs ($Padj < 0.05$; Table 3).

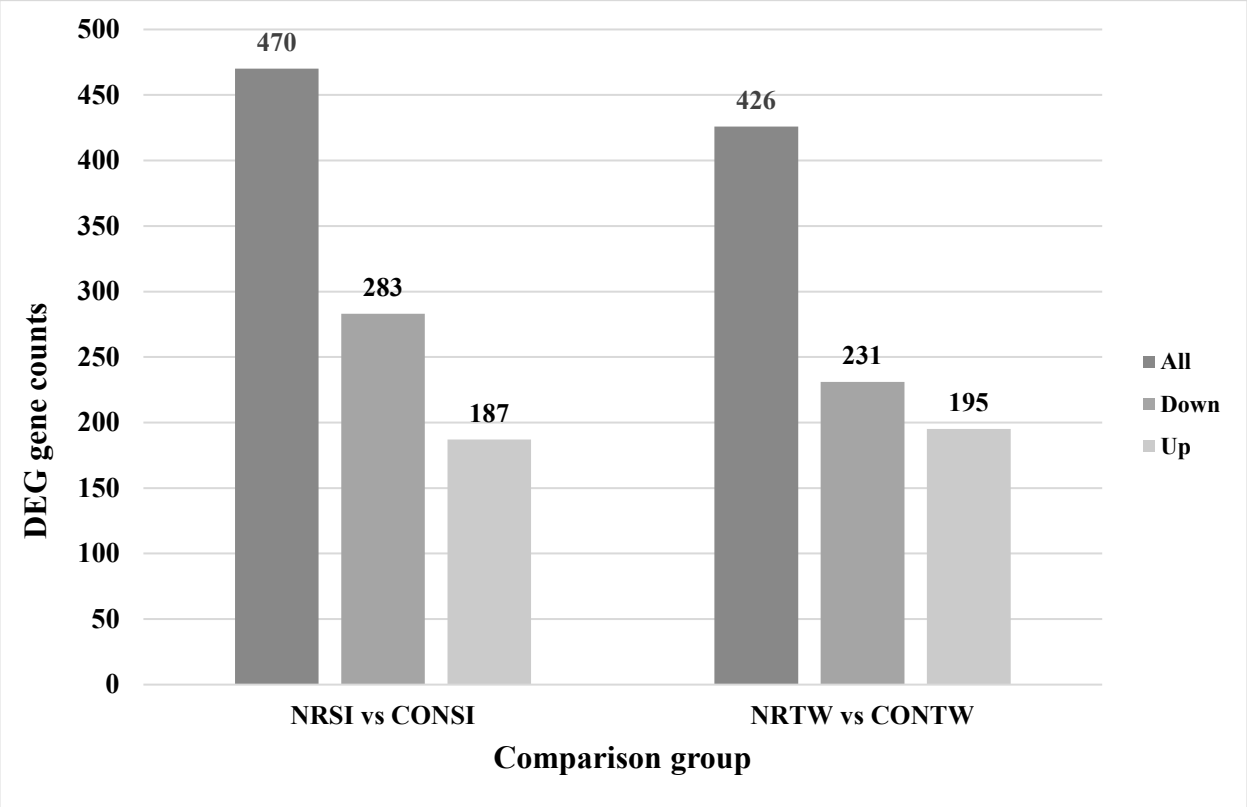

Figure 4: Cotyledon DEG counts between treatments based on number of lambs. Comparisons were made for nutrient restricted singletons (NRSI) vs control diet singletons (CONSI) and nutrient restricted twins (NRTW) vs control diet twins (CONTW).

Table 3: DEGs detected in both comparison groups. (*P-adj* < 0.09).

Gene ID	Gene Name	log2FoldChange	padj
NRSI vs CONSI			
ENSOARG00000006615	Poly [ADP-ribose] polymerase (W5P9H5_SHEEP)	5.69	0.083
ENSOARG00000000908	Cytochrome P450 aromatase (W5NRZ8_SHEEP)	-6.88	0.059
novel.1102		-20.68	0.00008
NRTW vs CONTW			
	None		

KEGG and GO analyses

Seventeen KEGG pathways ($P < .05$) were represented in DEGs between cotyledons from singleton pregnancies in the NR vs CON groups (Figure 5). Those of most interest include pyruvate metabolism (4 DEGs), antigen processing and presentation (7 associated DEGs), and extracellular matrix receptor interactions (6 associated DEGs). Fourteen KEGG pathways ($P < 0.05$) were represented by DEGs from cotyledons collected from twin pregnancies of NR vs CON ewes. Those of interest include protein digestion and absorption (9 associated DEGs), extra-cellular matrix interaction (5 associated DEGs), and antigen processing and presentation (5 associated DEGs; Figure 6).

Gene Ontology analysis identified 32 pathways containing DEGs from cotyledons from NR singletons vs CON singletons including immune response, immune system process, and G-protein-coupled receptor signaling pathway ($P < 0.05$; Table 7). Analysis of DEGs between the twins from the NR group and CON group identified four GO, including cell surface receptor signaling pathway, oxidation-reduction process, and proteolysis (Figure 8; $P < 0.05$).

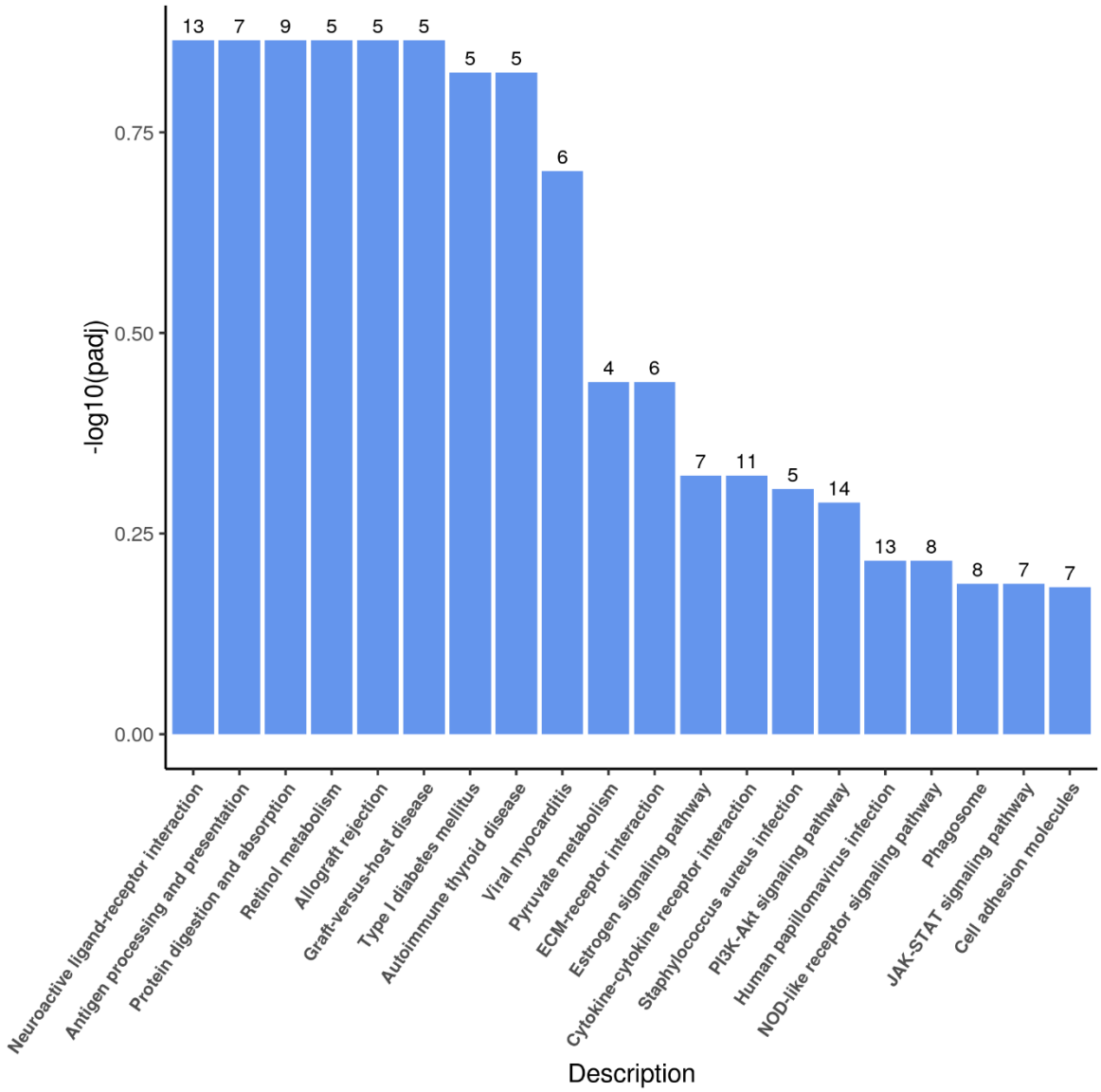

Figure 5: KEGG pathways identified from DEGs in cotyledons from NR vs CON singleton pregnancies. Numeric values denote the number of differentially expressed genes. KEGG pathways are listed by level of significance, $P < 0.05$.

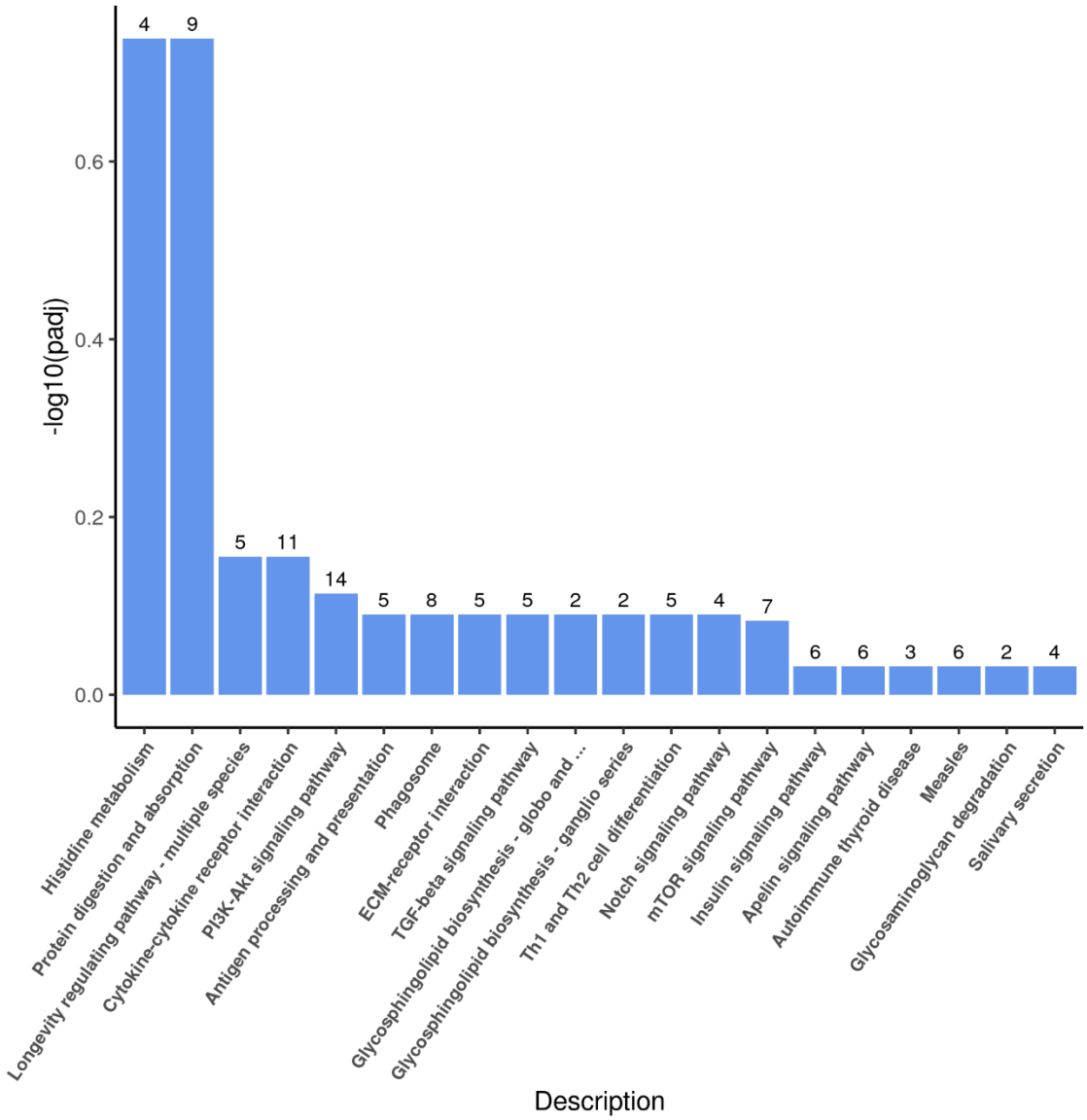

Figure 6: KEGG pathways identified from DEGs in cotyledons from NR vs CON twin pregnancies. Numeric values denote the number of differentially expressed genes. KEGG pathways are listed by level of significance, $P < 0.05$.

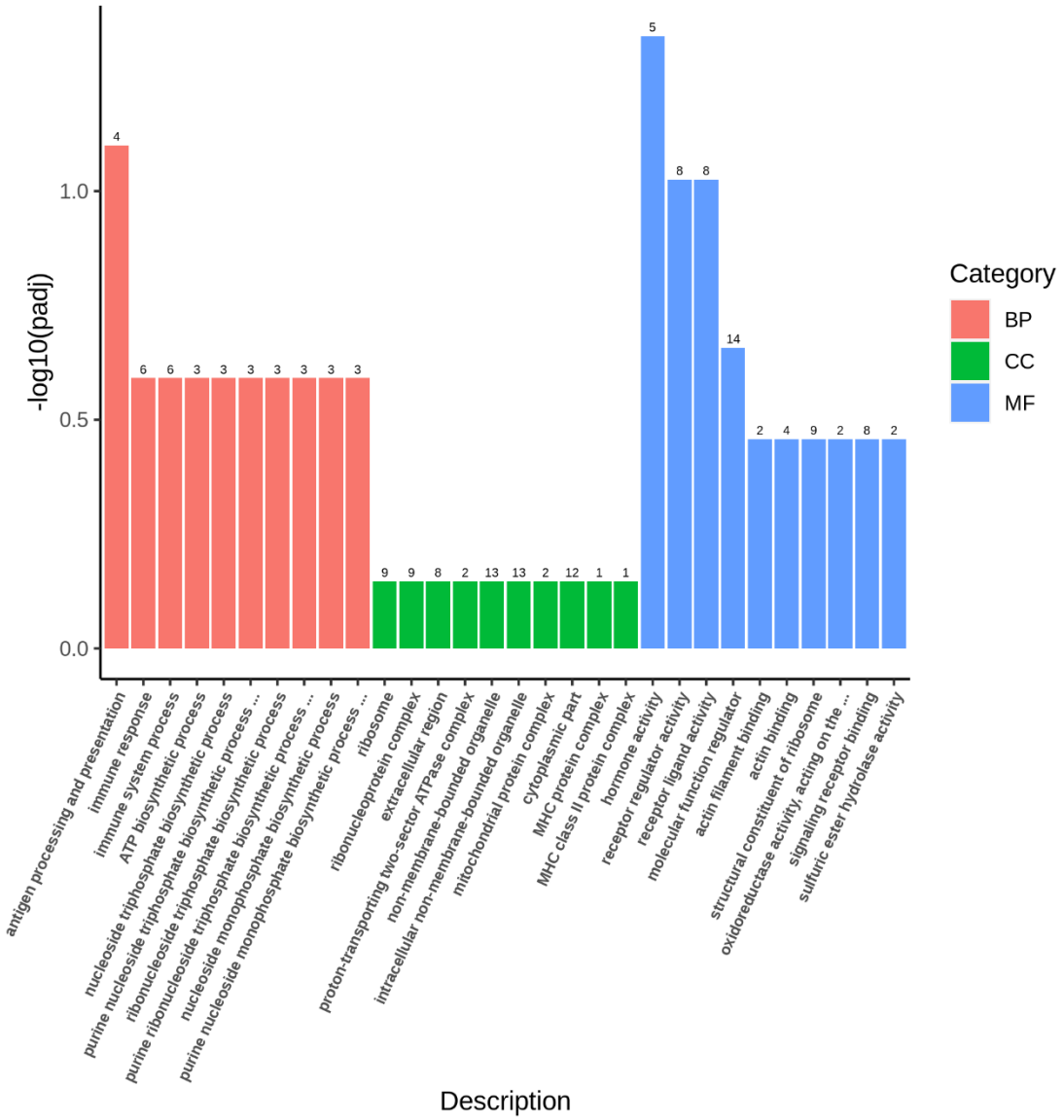

Figure 7: Gene ontology term represented by DEGs from NRSI vs CONSI cotyledons. Numeric values denote the number of differentially expressed genes. Terms are listed based on significance within category (BP, biological process; CC, cellular components; MF, molecular function), $P < 0.05$.

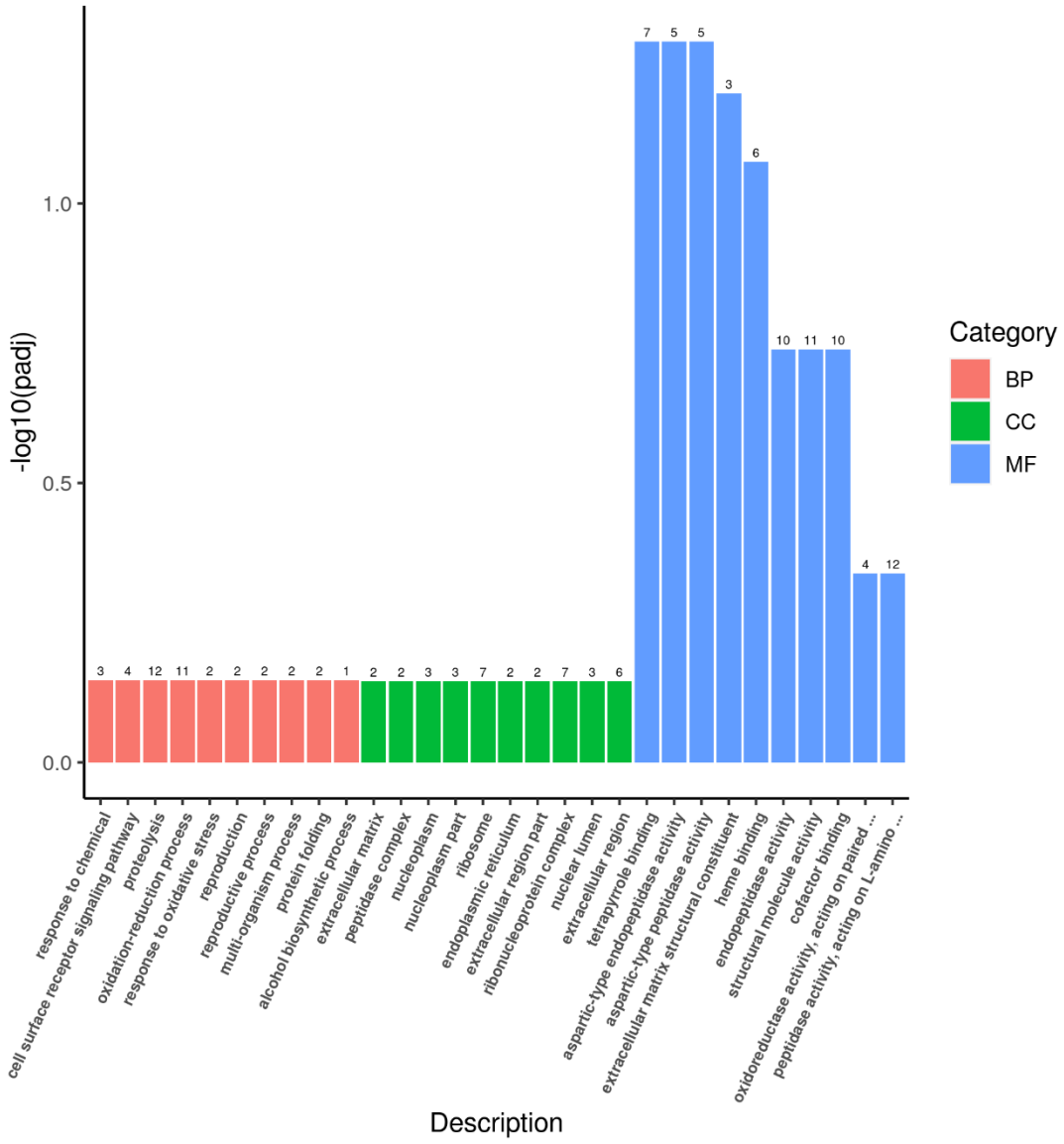

Figure 8: Gene ontology term represented by DEGs from NRTW vs CONTW cotyledons. Numeric values denote the number of differentially expressed genes. Terms are listed based on significance within category (BP, biological process; CC, cellular components; MF, molecular function), $P < 0.05$.

Discussion

Drought, limited forage availability, and nutrient restriction are conditions that sheep raised on rangelands frequently face. These conditions have negative effects on fetal and offspring development. The placenta plays a vital role in feto-maternal nutrient exchange. Placental efficiency and nutrient transfer capabilities can affect the health and performance of offspring throughout their lives.

Ewe body weights began to differ starting on day 72 of gestation and differences remained through lambing with the CON ewes being heavier than their NR counterparts. The difference in ewe body weights between treatment groups corresponds with the period of increased fetal growth and nutrient requirements. Day 90 of gestation is when fetal lamb growth dramatically increases (Redmer et al., 2004). The rapidly growing fetus has an increased requirement for essential molecules, like glucose, oxygen, and amino acids (Hay, 2006). Dam nutrient requirements also increase during this period, by 30-50% (Caton et al., 2019a). The increase in both fetal and maternal nutrient requirements explain the NR ewe weight loss over this later portion of gestation. Nutritional requirements during the early part of gestation are near maintenance and this is due to minimal fetal growth, this early period is dedicated to the development of the placenta. This lower nutrient requirement likely explains why there was not a difference in ewe body weight during the first trimester.

There was no difference in lamb birth weight in this trial. Historically, offspring birth weight is used to evaluate fetal programming effects and as a measure of offspring future performance. In lambs, however, birth weights may not be an adequate tool to evaluate dam health, placental function, lamb longevity, or nutritional status. This is likely because changes in

lamb birth weights are difficult to induce unless nutrient restriction is severe. Past work done with varying levels of nutrient restriction during different periods of gestation resulted in contradicting results in lamb birth weights (Heasman et al., 1998; Meza-Herrera et al., 2015; Vasquez-Hidalgo et al., 2022), and this is due to the timing and duration of nutritional insult. Slight levels of nutrient restriction or early periods of restriction typically do not result in changes to lamb birth weights, but often results in changes to the placenta, specifically at the site of fetal attachment (Field et al., 2015). In trials where ewes experienced chronic restriction throughout pregnancy, a shift in placentome shape has been observed (Vonnahme et al., 2006). Placentomes contain more fetal tissue and increased fetal capillaries in response to low nutrient availability (Vonnahme et al., 2006; Hafez et al., 2010) Although an increase in fetal tissue would increase surface area attachment, work in ewes evaluating placentome shape and angiogenic related factors reported that placentome vascularization may be more related to size and weight rather than shape or morphology (Vonnahme et al., 2008). However, placentome shape is related to placentome weight. Placentomes with the least amount of fetal tissue tend to be smaller and lighter (Vatnick et al., 1991a; Vonnahme et al., 2006; Vonnahme et al., 2008). In this study placentome shape was not recorded, however, total placental weight and cotyledon weights were.

There was no change in total placental weight resulting from dietary insult. Placental weight and fetal weight are highly correlated (Heasman et al., 1998). This correlation may explain why there was no difference in placenta weight between treatment groups. However, there is evidence that supports the placenta underwent changes to minimize fetal effects. In the present study, the weight of individual cotyledons from the CON group were heavier than those

from the NR group. This weight difference indicates that cotyledons from the NR group are not as highly vascularized, which would decrease the capacity for nutrient transfer (Vonnahme et al., 2008). Originally, it was hypothesized that cotyledons from the NR group would be heavier than those of the control which may indicate a dramatic increase in vascularization, and placental adaptations to maternal nutrient restriction. Interestingly, the opposite occurred. The treatments applied in this study represented chronic nutrient restriction, meaning the placenta was likely unable to fully compensate. This ability of the placenta to adapt is observed in trials where acute restriction is applied (Steyn et al., 2001; Ma et al., 2011; Field et al., 2015). Ewes experiencing restriction during the first half of gestation yielded heavier placentas (Heasman et al., 1998). Protein restriction in rats during early gestation placentas has yielded heavier placentas (Langley-Evans et al., 1996). The reallocation to a maintenance diet provides the placenta an opportunity to rapidly drive placental growth to support fetal development. Ewes in the present study did not have this opportunity, which may explain the differences in cotyledon weights.

Placental vascularization is a highly regulated process sensitive to maternal and fetal signaling. A complex system of capillaries dictates the transfer of essential nutrients and gas exchange. Fewer capillary numbers limits the ability for nutrient and oxygen to transfer (Pearce et al., 2016). Following RNA-sequencing analysis, common themes were detected between the DEGs and biological pathways expressed by KEGG and GO databases. These themes involve hormone synthesis, cotyledon vascularization, the involvement of immune related cells within the placenta, and the behavior of cells in relation to adhesion, migration, vascularization of the placenta, and placental metabolism.

There were no DEGs detected at the selected adjusted *p-value* when the comparison between NR twins and CON twins was made. This is likely a result of the natural nutrient partitioning that occurs in twinning. Twin pregnancies have a higher nutrient requirement but may be more sensitive to maternal and placental adaptations during nutrient restriction (Blickstein, 2004; Cleal et al., 2007). When twin lambs experienced nutrient restriction during early to mid-gestation until day 70 of gestation, birth weights do not differ from those of singletons. (Cleal et al., 2007). This is likely due to the timing of restriction and the compensatory nature of the placenta. The number of fetuses does not affect the density of vasculature within the cotyledon but decreases vasculature volume (Pant et al., 2003; Wirrenga et al., 2004; Reynolds et al., 2005b). The number of fetal capillaries does not change, therefore the ability to exchange nutrients and gas is not impacted. The size of capillaries is decreased in twin pregnancies, limiting the amount of blood and nutrients that are transferred. There were several biological pathways detected in the comparison of twin lambs from this trial related to BNC migration and cotyledon vascularization that were impacted by maternal nutrient restriction. The lack of DEGs detected may be the result of the twin placenta being able to adapt to maternal nutrient restriction in early gestation; therefore, not requiring the transcriptional adjustments to support proper fetal development in later gestation that were observed in singleton placentas. The natural changes that occur to the placenta because of fetal number may be preventing these transcriptional changes from being detectable.

RNA-sequencing analysis from this trial identified several DEGs and biological pathways involved in hormone – receptor interaction. Estrogen signaling was disrupted in cotyledons from NR singletons compared to CON singletons. KEGG analysis identified seven DEGs associated

with the estrogen signaling pathway; three up-regulated and four down-regulated in the NR singleton pregnancies. Estrogen has important functions in general reproductive physiology, but also plays a role in fetal development. Estrogens are involved in uterine gene expression during cyclicity and are responsible for the activation of estrogen receptor (ESR), progesterone receptor (PGR), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sahlin and Eriksson, 1996; Zou and Ing, 1998; Ing and Zhang, 2004) There is evidence that when estrogen binds to ESR, it induces vasodilatory action and induces angiogenesis (Pastore et al., 2012; Mayra et al., 2014; Reynolds et al., 2015; Bunma et al., 2020). Decreased levels of estrogen during pregnancy is tied to preeclampsia, indicating estrogen's role in placental vascularization (Berkane et al., 2017; Shu et al., 2021). Estrogen can also bind to transmembrane G-protein coupled receptor 30 (GPR30) which causes rapid vasodilation (Fredette et al., 2018). Genes associated with G-protein coupled receptor pathways were up and down-regulated in NR cotyledons from singleton pregnancies. Furthermore, cytochrome P450 aromatase (*CYP*) was identified as a DEG in cotyledons from NR singletons and was down-regulated. Interestingly, in the same comparison (NR singletons vs CON singletons) a disruption to the estrogen signaling pathway was detected following KEGG analysis. Although *CYP* was not an identified DEG in NR twin cotyledons, cytochrome P450 4B1 was identified when GO biological processes were analyzed. However, in this group of cotyledons, *CYP* was up-regulated as a result of diets. *CYP* is the limiting step in estrogen synthesis (Bondesson et al., 2015). A down-regulation in NR singletons may disrupt estrogen production. Gestating rats that experienced decreased placental blood flow had changed expression of *Cyp* in placental tissues indicating a decrease in circulating estrogen levels (Vonnahme et al., 2001; Maliqueo et al., 2016; Shin et al., 2021). A reduction in estrogen

synthesis paired with a disruption in estrogen's affinity to its receptors, in response to dietary stress, provides more evidence to support that nutrient restriction is causing changes to placental function. Estrogen's involvement in angiogenesis could also explain why cotyledons from NR ewes were lighter.

Pathways involved in antigen processing and presentation were disrupted in NR cotyledons from both singleton and twin pregnancies. One of the major functions of the placenta is acting as a partial immunological barrier between the maternal and fetal systems. Half of the DNA in an embryo or fetus is comprised of paternal DNA, which is foreign to the maternal system. Rather than the maternal immune system being down-regulated, it is able to recognize and tolerate the foreign fetal cells (Golos et al., 2010). There were 5 immune-related DEGs detected in cotyledons collected from twin pregnancies and 7 identified in cotyledons from singletons pregnancies detected in KEGG pathways. All 12 DEGs0 were downregulated in cotyledons from NR ewes. The affected pathway is involved in major histocompatibility complex (MHC) I and II expression. MHC I is expressed on the surface of any epithelial cell, providing system wide antigen presentation, and is also involved in T cell receptor signaling pathway and natural killer cell (NK) cytotoxicity (Nanda et al., 2006). MHC II molecules are located on the surface of antigen-presenting cells, like dendritic cells and macrophages, and are involved in T cell signaling, but more specifically cytokine production and the activation of other immune cells initiated by T cell signaling (Nanda et al., 2006). The fast-growing and invasive behavior of the placenta has been compared to that of cancer cells. As tumors develop, they down-regulate MHC I, decreasing antigen presentation (Cornel et al., 2020). Tumors that inhibit MHC expression are often more aggressive and metastasize (Taylor and Balko, 2022). This

behavior is associated with increased cell proliferation, adhesion, and invasion (Watson et al., 2006; Park et al., 2018). Furthermore, the role of BNCs in ruminant placentas is to adhere and invade into the uterine endometrium to aid in nutrient transport. Ruminant trophoblast cells display MHC molecules (Davies et al., 2000; Rutigliano et al., 2016). A down-regulation in the MHC expression in NR cotyledons indicates that trophoblast cells are becoming more aggressive, increasing their migratory behavior in an attempt to limit fetal nutrient restriction.

Another function of MHC is the recruitment of NK cells. In human cancer research, the downregulation of MHC I makes cancer cells more susceptible to detection by NK cells (Bubeník, 2003). Uterine natural killer cells (uNK) are a normal part of healthy pregnancy and these cells produce cytokines and angiogenic factors essential for the maintenance of pregnancy (Gaynor and Colucci, 2017). Trophoblast cells present ligands that are act on NK cell receptors (Sharma, 2014; Mahajan et al., 2022). This communication between trophoblast cells and uNK is involved in maternal immune tolerance (Sharma, 2014; Mahajan et al., 2022). It is possible NR pregnancies down-regulated genes associated with MHC to increase recruitment of uNK. An increase in uNK could increase cytokine and angiogenic factor release (Chen et al., 2017). Work *in vitro* determined that NK cells release angiogenic related factors such as angiopoietin-1,-2 (*Ang-1 -2)*, which are present in the placenta and used as markers to evaluate placental vascularization (Robson et al., 2019). The recruitment of uNK may be an attempt to improve vascularization in the NR cotyledons. This again provides some evidence to the placenta's attempt to minimize the effects of dietary restriction.

Disruptions in cytokine-cytokine receptor interactions were detected in the cotyledons from twins and singletons from NR ewes. In cotyledons from twins, there were 4 up-regulated

and 7 down-regulated DEGs, and in the singletons, 2 genes were up-regulated and 9 were down-regulated in NR cotyledons. Cytokines have several regulatory activities and are released by cells in response to an immune response or hormone signaling (Zhang and An, 2007). Prolactin (PRL) is a notable cytokine that was downregulated in NR cotyledons from both singleton and twin pregnancies. PRL is involved in maternal metabolic changes and glucose partitioning to the fetus during gestation (Sorenson and Brelje, 1997; Baeyens et al., 2016). In human placentas, the expression of *PRL* is tied to trophoblast cell migration and invasion into the maternal endometrium (Stefanoska et al., 2013). There is also evidence that *PRL* inhibits pro-inflammatory cytokine action (Olmos-Ortiz et al., 2019). Pro-inflammatory cytokines in the placenta are correlated with diagnosis of preeclampsia in women (Aggarwal et al., 2019). Suppression of *PRL* expression could be associated with a decrease in BNC migration and an increase in the presence of pro-inflammatory cytokines. This further supports the idea that cotyledons from NR ewes experience a hypoxic intrauterine environment. A reduction in *PRL* could also indicate a reduction in BNC function and migration, which may affect nutrient transfer across placental membranes.

A cytokine receptor of note that was down-regulated in NR twins is bone morphogenetic protein receptor type 2 (*BMPR2*). This receptor is involved in bone and cartilage formation but is also involved in cell proliferation and regulated apoptosis. Cell proliferation and apoptosis are normal biological functions of the cell cycle, and these processes are highly involved in placentation and fetal growth. Dysregulation in cell turnover is associated with preeclampsia (Heazell et al., 2006). A downregulation in *BMPR2* limits the binding of specific cytokines which include growth differentiation factor (GDF) – 2, 5, 6, 7, and 9. GDFs are members of the

transforming growth factor-β subfamily (*TGFβ*) (Sugulle et al., 2009). *TGFβ* is involved in the regulation of trophoblast cell invasion by decreasing cell proliferation and increasing the rate of apoptosis (Lash et al., 2005; Jones et al., 2006). A reduction in *TGFβ* expression as a result of decreased binding of *BMPR2* would indicate an increase in BNC migratory behavior. An increase in this migratory behavior would allow for more efficient nutrient transfer to the fetal system. This may be another mechanism by which NR cotyledons are adjusting their function to support fetal growth.

Metabolism is an important process in placental function and proper placental metabolism is essential for fetal health. Pathways of interest in singleton pregnancies from KEGG analysis include pyruvate metabolism, type 1 diabetes mellitus, and protein digestion and absorption. Pyruvate is a molecule produced from the breakdown of glucose and then is involved in the production of adenosine triphosphate (ATP). Four genes were associated with pyruvate metabolism in the singleton pregnancies, 3 were up-regulated while 1 was down-regulated in NR pregnancies. A dysregulation in pyruvate metabolism could lead to changes in ATP production. However, the general up-regulation of associated genes may indicate that there is an increase in ATP production, which is then used by the placenta (Sferruzzi-Perri et al., 2019). *Pyruvate kinase* was a notable gene that was up-regulated, which is directly involved in the process of glycolysis and the production of ATP. Not all nutrient transfer across the placenta is achieved by passive diffusion. Energy is required by the placenta to transfer large molecules, like amino acids into the fetal compartment. The dietary restriction experienced by the placenta in this trial led to an up-regulation in genes associated with pyruvate metabolism. It is speculated that the placenta may be attempting to increase the diffusion of nutrients into the fetal compartment. Increased

diffusion of nutrients, especially large nutrients such as amino acids, vitamins, and minerals, increases the placentas energy requirement, causing a greater need for ATP production.

Insulin resistance, a precursor to diabetes, is a condition that is difficult to induce in fetal lambs. This may be because of the placentomes ability to change vascular components to limit fetal affects. However, in singleton pregnancies 5 genes associated with type 1 diabetes mellitus were all down-regulated in NR singleton cotyledons. Type 1 diabetes is classified as an autoimmune disease and has been associated with mis regulation of MHC molecules and the death of pancreatic beta cells (Durinovic-Bello, 1998; Cucca et al., 2001; Majali-Martinez et al., 2021). Fetal lambs subjected to placental insufficiencies while *in utero* have decreased insulin production (Thorn et al., 2012). Nutrient restriction during pregnancy limits circulating glucose, therefore inducing a hypoglycemic environment. Research done evaluating human trophoblast cells behavior in a hypoglycemic environment severely reduces the number of trophoblast cells but increased their migratory behavior (Majali-Martinez et al., 2021). We speculate that the type 1 diabetes pathway detected in singleton pregnancies has a similar effect on the population of BNCs in NR cotyledons. The lack of glucose reaching the fetus may promote BNC invasion, as supported by the previously discussed MHC and BMPR2 data but could also reduce cell numbers. An up-regulation in cell migration would increase the efficiency of nutrient transfer, but the reduction in cell population may counteract the effects of increased cell migration.

Type 1 diabetes is also associated with a dysregulation of nitric oxide (NO) production (Fowler, 2008). Nitric oxide synthase (NOS) was differentially expressed in twin cotyledons from NR ewes, and this gene was down-regulated in response to diet. NOS is produced by placental tissue and is involved in maintaining low vascular resistance (Al-Hijji et al., 2003).

Although, the metabolic pathway for type 1 diabetes was not detected in cotyledons from the twin group, the down-regulation of *NOS* expression may indicate that these twins are experiencing some level of hypoglycemia because of maternal dietary stress. A reduction in NOS may also lead to constriction on fetal and placental vasculature, exasperating fetal nutrient restriction and oxygenation. A deficiency in NOS does not prevent placental vascularization, but NOS is required for adequate vessel formation (Ortiz and Garvin, 2003; Tsutsui et al., 2009). A reduction in NOS expression may indicate that the vascularization in the NR cotyledons is not as highly developed as in the CON counterparts. This limitation in vascularization can result in fetal hypoxia, ultimately affecting fetal development.

Amino acids are essential for fetal growth, and during pregnancy amino acid concentrations in fetal blood are higher than in the maternal system (Cetin et al., 1992). This indicates that there is increased transfer across the placenta. Protein digestion and absorption in twin pregnancies had 8 down-regulated genes and 1 up-regulated gene in NR ewes. In the singleton pregnancies there were 6 genes down-regulated and 3 genes up-regulated. Notable genes that were down-regulated in the NR singletons and twins included *PAG-1* and *-3*. *PAG-9* was down-regulated in the cotyledons of NR twins. PAGs are often used by producers to diagnose pregnancy and assess viability. *PAG -1, -3,* and *-9* are solely expressed from BNCs through the syncytium (Green et al., 2000). A down-regulation in their expression indicates that BNC migration may be reduced from nutrient restriction. This down-regulation may also be indicative of decreased placental health and a sign that NR pregnancies are as functional as their CON counterparts. The research done assessing the effect that maternal nutrient restriction has on PAG expression and BNC function is limited. Work in an obese ewe model reported altered

PAG expression in ewes following exposure to overnutrition (McCoski et al., 2018). While the function of PAGs remains to be determined, these data suggest BNCs function is sensitive to dietary stress.

Implantation is a key component in placental development and early embryonic development. Embryonic implantation and placentation are events that must occur at specific timepoints, and there is specific cell signaling and cross talk occurring between the uterine endometrium and trophoblast cells. A DEG of interest that was up-regulated in the cotyledons from NR singleton pregnancies is poly [ADP-ribose] polymerase (*PARP*). *PARP* is often associated with tissue remodeling and regulated apoptosis and is involved in the maintenance of the cell cycle (Simbulan-Rosenthal et al., 2000; Pagano et al., 2007). A relationship between *PARP* and uterine tissue remodeling and cell differentiation and proliferation has been established (Demir et al., 2002; Joswig et al., 2003; Correia-da-Silva et al., 2004). Researchers observed that during implantation *PARP* expression was elevated at implantation sites in mice (Joshi et al., 2014). *PARP* is essential for pregnancy establishment, with knockout mice experiencing early embryonic loss following implantation failure (de Murcia et al., 1997; Ménissier de Murcia et al., 2003). A up-regulation in *PARP* expression in human cancer research is correlated with aggressive tumor behavior (Donizy et al., 2020). *PARP* has been detected in mice and is involved in the differentiation of trophoblast cell (Hemberger et al., 2003). In this trial, an up-regulation in expression could indicate an increase in BNC proliferation and migration in cotyledons from NR ewes. BNCs migrate into maternal caruncular tissue, making nutrient transfer to the fetus more efficient. An increase in *PARP* may indicate that the lack of available nutrients triggers an increase in cell proliferation and migration into the uterine

endometrium; therefore, the placenta is attempting to increase nutrient transfer to the fetus. Furthering this idea, genes associated with extracellular matrix (ECM) receptor interaction were altered in the cotyledons from both singleton and twin pregnancies following nutrient restriction. In singleton cotyledons there were 5 down-regulated genes and 1 up-regulated in NR ewes. Cotyledons from NR twin pregnancies have 5 ECM-related genes expressed, all down-regulated. The ECM and the interaction with its receptors is involved in regulating cell behavior, communication between cells, cell adhesion and migration (Nguyen-Ngoc et al., 2012; Plotnikov et al., 2012; Schlie-Wolter et al., 2013; Lange et al., 2014) There are several proteins and their receptors that make up the ECM. Thrombospondins (*THBS*) is a glycoprotein that was down-regulated as a result of dietary stress in twin pregnancies. *THBS* regulates the migration and invasion of cells and is a regulator of angiogenesis, functions that are all essential in placental formation (Lawler and Detmar, 2004; Greenaway et al., 2007b; Garside et al., 2010). The involvement of *THBS* in vascular development is inhibitory, made apparent in knock-out mice (Wang et al., 2003; Sund et al., 2005; Greenaway et al., 2007a). Without the expression of THBS, vascularization in mice was increased and epithelial cells could respond to angiogenic related factors, such as *VEGF* (Wang et al., 2003; Sund et al., 2005; Greenaway et al., 2007a). Down-regulation of *THBS* expression indicates that there is a need for increased vasculature. During periods of dietary insult, there is a massive reliance on vasculature to transfer nutrients. The down-regulation of this gene expression indicates that the vascular network in the placenta is undergoing changes in order to compensate for fetal nutrient demands.

The phosphatidylinositol 3-kinase- protein kinase B (PI3K-Akt) pathway is involved in metabolism, cell proliferation, and angiogenesis (Rodon et al., 2013). This signaling pathway

was represented by genes that were up- and down-regulated in NR cotyledons from the singleton and twin pregnancies. A dysregulation in this pathway is commonly associated with aggressive types of cancer (Karami fath et al., 2022). The role of PI3K-Akt in the placenta is similar to that in cancer cells, in that it increases cell proliferation and vascularization. An inhibition of PI3K-Akt signaling in human placentas repressed trophoblast cell invasion (Zhu et al., 2016). There are many receptors and ligands that make up this signaling pathway, but the down-regulation of integrin β 1 (*ITB1*) in the NR cotyledons of twins and the down-regulation of ECM in singleton NR cotyledons is important to note. *ITB1* is directly involved in cell migration as it acts directly on matrix metalloproteinases (MMP), which are involved in the breakdown and remodeling of the ECM of tissues (Visse and Nagase, 2003). An increase in *ITB1* up-regulates the expression of MMPs, which allows for increased cell invasion (Grafinger et al., 2020). The decreased expression of *ITB1* may indicate a decreased expression of MMPs, therefore affecting BNC migration into the endometrium. This dysregulation is interesting in the fact that other notable pathways and expression of individual genes promote cell invasion to aid in nutrient transfer. This disruption may provide evidence that nutrient availability can negatively affect BNC behavior and function; therefore, decreasing placental efficiency.

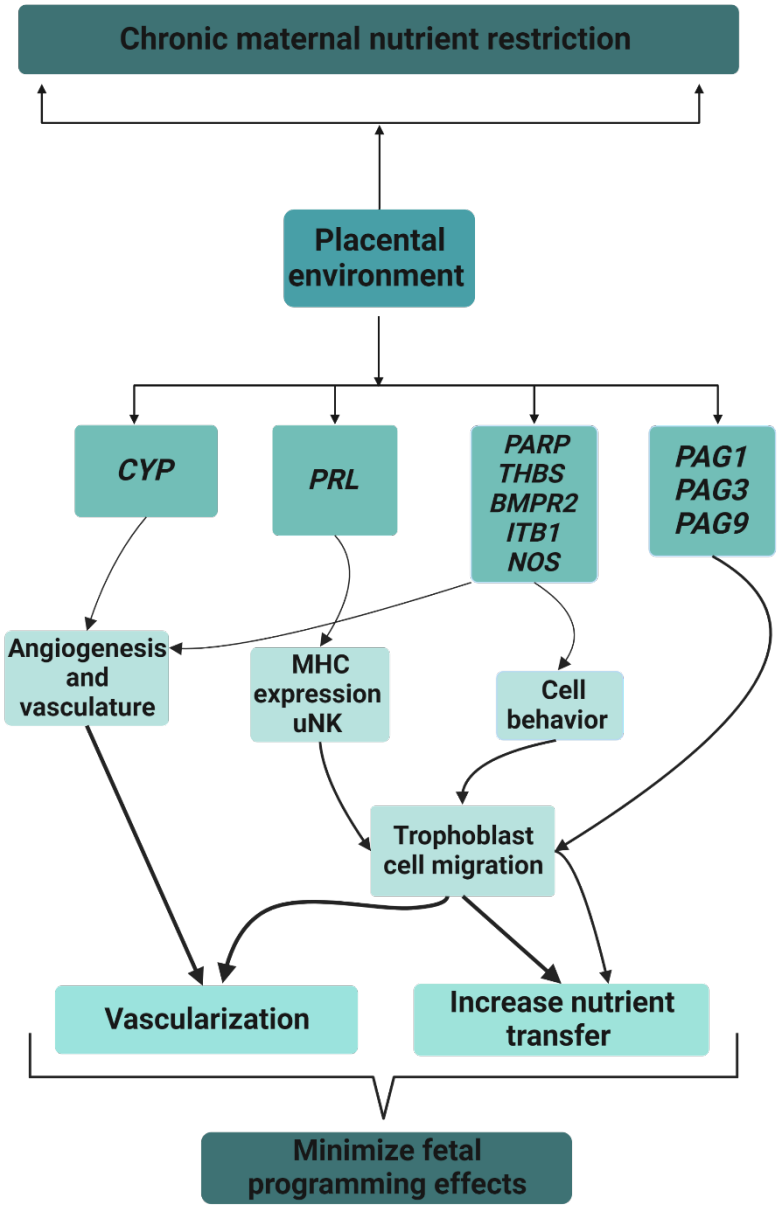

Figure 9: Interaction of notable DEGs with roles in trophoblast cell development, placental vasculature, and immune function of the placenta.

Conclusion

There are several factors involved in placental function, and it is well known that dietary insults lead to alterations in placental function. This study sheds light on the mechanisms behind placental adaptation in response to maternal restriction. BNC migratory behavior allows for the efficient transfer of nutrients during pregnancy. Without this migration, the physical distance that nutrients need to travel is greater, and this distance severely limits the transfer of essential molecules, especially large molecules like glucose and amino acids. There is limited research on how BNCs respond to maternal dietary restrictions; however, the results of this study suggest that dietary stress alters cotyledon gene expression. It is important to note that RNA-sequencing is a snapshot of genes being actively transcribed at the time of tissue harvest. Gene expression changes through gestation. The diets applied in this trial provide information on the effects of chronic nutrient restriction on cotyledon development and function (Figure 9). This RNA-sequencing data revealed several pathways and genes within the placenta that increase cell invasion, but also several processes that negatively affect placental cell behavior by limiting cell migration and invasion. These data indicate that cell migration and invasion are suppressed in the cotyledon of NR ewes, and secondary mechanisms may be activated to compensate. Further work is needed to confirm this hypothesis.

Diet was designed to mimic the conditions that ewes raised in native rangelands may experience during gestation. The practicality of the diets selected for this study provided results that may be faced by sheep producers. Even though there were no changes to lamb birth weights or placental weight, there were changes to cotyledon gene expression. Collectively, this work identifies several pathways involved in cotyledon development and function disrupted in NR

ewes, as well as several potential compensatory mechanisms that may rescue placental function. Future work should include evaluation of offspring health. This may serve as an assessment of the placenta's ability to truly compensate for poor maternal nutrition. Additionally, observing lamb morbidity, survivability, and organ weights would be valuable information to evaluate fetal programming effects and how lamb future health could be affected by the changes in cotyledon behavior and function. Lastly, future work may focus on strategic supplementation protocols that can be used to support cotyledon development and function during times of dietary stress.

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